iontorrent

Ion AmpliSeq™ Library Kit Plus

USER GUIDE

for use with:
Ion Torrent™ Dual Barcode Kit 1–96
IonCode™ Barcode Adapters 1–384 Kit
Ion Xpress™ Barcode Adapters Kits
Ion Library Equalizer™ Kit
Ion AmpliSeq™ On-Demand Panels
Ion AmpliSeq™ Ready-to-Use Panels
Ion AmpliSeq™ Made-to-Order Panels
Ion AmpliSeq™ Community Panels
Ion AmpliSeq™ Sample ID Panel

Catalog Numbers 4488990, A35907, A38875
Publication Number MAN0017003
Revision E

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

ThermoFisher SCIENTIFIC

Life Technologies Corporation |
5781 Van Allen Way |
Carlsbad, California 92008 USA

Products manufactured at this site:

- Ion AmpliSeq™ Library Kit Plus
- Ion Torrent™ Dual Barcode Kit 1–96
- IonCode™ Barcode Adapters 1–384 Kit
- Ion Xpress™ Barcode Adapters Kits
- Ion Library Equalizer™ Kit

Life Technologies Corporation |
6055 Sunol Blvd |
Pleasanton, California 94566 USA

Products manufactured at this site:

- Ion AmpliSeq™ panels

For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: MAN0017003 F (English)

Revision	Date	Description
E	27 January 2025	Corrected the quantity per well and number of reactions in the Ion Torrent™ Dual Barcode Kit 1–96. See "Ion Torrent™ Dual Barcode Kit 1–96" on page 12.
D.0	2 January 2024	• Reorganized chapters 1, 2, and 3 into 5 chapters. • Added support for MagMAX™ Pure Bind Beads for purification. • Added appendix information about using panels.
C.0	5 November 2018	• Support added for Ion 510™ and Ion 550™ Chips. • Added Ion AmpliSeq™ Library Kit Plus 384-reaction size (Cat. No. A38875). • Support added for Ion GeneStudio™ S5 Systems. • Support added for Ion Torrent™ Dual Barcode Kit 1–96. • Protocol updates.
B.0	17 October 2017	• Product information table updated with new Ion AmpliSeq™ On-Demand Panel configurations. • Added Ion AmpliSeq™ Library Kit Plus 96-reaction size (Cat. No. A35907). • "Expand any Ion AmpliSeq™ panel with a spike-in panel" moved from Appendix D to Appendix E.

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

Contents

1 Product information

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

Product description

The Ion AmpliSeq™ Library Kit Plus is used with Ion AmpliSeq™ panels to manually prepare amplicon libraries for Next–Generation Sequencing (NGS). The kit includes reagents to prepare libraries manually from DNA or RNA for sequencing on the Ion GeneStudio™ S5, Ion PGM™, Ion Proton™, and Ion S5™ Systems.

Key features of the kit include the following.

- Plate–based format for easier sample tracking.
- Barcoded sample multiplexing enables amplicon libraries to be combined and sequenced on a single chip.

Note: The Ion AmpliSeq™ Kit for Chef DL8 (Cat. No. A29024) is used with Ion AmpliSeq™ panels for the automated preparation of amplicon libraries for Next–Generation Sequencing (NGS) using the Ion Chef™ System. See the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432).

Bisulfite analysis

Bisulfite analysis can be performed with modifications to the standard Ion AmpliSeq™ Library Kit Plus. See the *Bisulfite Methylation Library Production using the Ion AmpliSeq™ Library Kit Plus User Bulletin* (Pub. No. MAN0017662) or the *Bisulfite Methylation Library Production using the Ion AmpliSeq™ Kit for Chef DL8 User Bulletin* (Pub. No. MAN0017892).

Tecan™ Freedom EVO™ liquid handling platform

For information about preparing Ion AmpliSeq™ libraries on the Tecan™ Freedom EVO™ liquid handling platform, see *Prepare Ion AmpliSeq™ Libraries using the Tecan™ Freedom EVO™ NGS Workstation User Bulletin* (Pub. No. MAN0010822), available at **thermofisher.com**.

Contents and storage

Ion AmpliSeq™ Library Kit Plus

The Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990) provides reagents for manually preparing 24 libraries for 1- or 2-pool panels (16 libraries for 3-pool panels, and 12 libraries for 4-pool panels).

Component	Amount	Storage
	Cat. No. 4488990 (24 reactions)	
5X Ion AmpliSeq™ HiFi Mix (red cap)	120 µL	–30°C to –10°C
FuPa Reagent (brown cap)	48 µL	
Switch Solution (yellow cap)	96 µL	
DNA Ligase (blue cap)	48 µL	
25X Library Amp Primers (pink cap)	48 µL	
1X Library Amp Mix (black cap)	1.2 mL	
Low TE (white cap)	6 mL	15°C to 30°C[1]

[1] Can be stored at –30°C to –10°C

Ion AmpliSeq™ Library Kit Plus (96 and 384 reactions)

The Ion AmpliSeq™ Library Kit Plus (Cat. No. A35907) provides reagents for manually preparing 96 libraries for 1- or 2-pool panels (64 libraries for 3-pool panels or 48 libraries for 4-pool panels).

The Ion AmpliSeq™ Library Kit Plus (Cat. No. A38875) provides reagents for manually preparing 384 libraries for 1- or 2-pool panels (256 libraries for 3-pool panels, and 192 libraries for 4-pool panels).

Component	Amount		Storage
	Cat. No. A35907 (96 reactions)	Cat. No. A38875 (384 reactions)	
5X Ion AmpliSeq™ HiFi Mix (red cap)	480 µL	4 × 480 µL	–30°C to –10°C
FuPa Reagent (brown cap)	192 µL	4 × 192 µL	
Switch Solution (yellow cap)	384 µL	4 × 384 µL	
DNA Ligase (blue cap)	192 µL	4 × 192 µL	
25X Library Amp Primers (pink cap)	192 µL	4 × 192 µL	
1X Library Amp Mix (black cap)	4 × 1.2 mL	16 × 1.2 mL	
Low TE (white cap)	2 × 6 mL	8 × 6 mL	15°C to 30°C[1]

[1] Can be stored at –30°C to –10°C

Workflow

The following workflow shows how to manually prepare amplicon libraries using the Ion AmpliSeq™ Library Kit Plus and Ion AmpliSeq™ panels.

Ion AmpliSeq™ Library Kit Plus workflow

Isolate genomic DNA or RNA (page 19)

For a list of recommended kits, see "Recommended materials and equipment" on page 14.

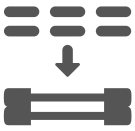
If starting with RNA, reverse transcribe the RNA to generate cDNA (page 30)

Amplify target regions from DNA (page 24) or cDNA (page 33)

With panels consisting of multiple primer pools, combine target amplification reactions after thermal cycling.

Partially digest amplicons with FuPa Reagent (page 25)

Ligate adapters with Switch Solution and DNA Ligase, then purify libraries (page 26)



Ion AmpliSeq™ Library Kit Plus workflow

Normalize or quantify the libraries

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. (page 40)
- Without further amplification, quantify libraries by qPCR and dilute to 100 pM. (page 45)
- Quantify libraries using the Qubit™ Fluorometer or the Agilent™ 2100 Bioanalyzer™ Instrument. (page 49)

(Optional) If barcode adapters are used, combine libraries before sequencing (page 75)

When barcode adapters are used, libraries can be combined in various ways before sequencing. Combining libraries maximizes chip use while minimizing cost and labor.

Proceed to prepare template, then sequence

Perform template preparation and sequencing as described in the instrument user guide.

Review plugin results and visualize variants

- Use the Ion Torrent™ ampliSeqRNA plugin or the coverageAnalysis plugin to view statistics and graphs that describe the level of sequence coverage produced for targeted genomic regions.

  For information about setting up plugins and performing data analysis with Torrent Suite™ Software, see the Torrent Suite™ Software help system or the *Torrent Suite™ Software 5.18 User Guide* (Pub. No. MAN0026163).
- Ion Reporter™ Software analyses are performed automatically when the data files are uploaded from Torrent Suite™ Software.

  For information about using the suite of bioinformatics tools in Ion Reporter™ Software to streamline variant analysis and reporting, see the Ion Reporter™ Software help system or the *Ion Reporter™ Software 5.20 User Guide* (Pub No. MAN0028322).

2 Reagents, supplies, and required materials

Required materials not supplied

In addition to a library kit and panel, you need the following materials and equipment. Unless otherwise indicated, all materials are available through **thermofisher.com**. Catalog numbers that appear as links open the web pages for those products.

Item	Source
One of the following thermo cyclers, or equivalent. • SimpliAmp™ Thermal Cycler • VeritiPro™ Thermal Cycler, 96-well • ProFlex™ 96-well PCR System • Applied Biosystems™ 2720 Thermal Cycler[1] • GeneAmp™ PCR System 9700 or Dual 96-well Thermal Cycler[1] • Veriti™ 96-Well Thermal Cycler[1]	 • A24811 • A48141 • 4484075 • 4359659 • Various • 4375786
One of the following. • Ion Library Equalizer™ Kit • Ion Library TaqMan™ Quantitation Kit and real-time PCR instrument • Agilent™ 2100 Bioanalyzer™ and Agilent™ High Sensitivity DNA Kit	 • 4482298 • 4468802 • Agilent G2939BA, Fisher Scientific™ 5067-4626
One of the following kits. • IonCode™ Barcode Adapters 1–384 Kit • Ion Xpress™ Barcode Adapters Kits • Ion Torrent™ Dual Barcode Kit 1–96	 • A29751 • Various • A39360
MicroAmp™ Optical 96-Well Reaction Plate with Barcode	N8010560, 4306737
MicroAmp™ Clear Adhesive Film	4306311
MicroAmp™ Optical Film Compression Pad	4312639

(continued)

Item	Source
Eppendorf™ DNA LoBind™ Microcentrifuge Tubes, 1.5-mL	Fisher Scientific™ 13-698-791
One of the following purification reagents. • MagMAX™ Pure Bind Beads (preferred) • Agencourt™ AMPure™ XP Reagent (optional)	 • A58521, A58522, A58523 • Fisher Scientific™ NC9959336, NC9933872
DynaMag™–96 Side Magnet, or other plate magnet	12331D
Nuclease-Free Water (not DEPC-Treated)	AM9932
Ethanol, Absolute, Molecular Biology Grade	Fisher Scientific™ BP2818500
Pipettors, 2–200 µL, and low-retention filtered pipette tips	**fisherscientific.com**
(RNA only) One of the following kits. • SuperScript™ IV VILO™ Master Mix • Ion Torrent™ NGS Reverse Transcription Kit • SuperScript™ VILO™ cDNA Synthesis Kit	 • 11756050 • A45003 • 11754050

[1] Supported but no longer available for purchase.

Ion Torrent™ Dual Barcode Kit 1–96

The Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360) provides 96 dual-matched barcode adapters in a convenient 96-well plate format. When used in combination with the Ion AmpliSeq™ Library Kit Plus or Ion AmpliSeq™ Library Kit 2.0, the barcodes enable multiple samples to be run on a single chip.

Component	Quantity	No. of reactions	Storage
Ion Torrent™ Dual Barcode Kit 1–96	1 × 96-well plate (6 µL/well)	96 (1 reaction per barcode)	–30°C to –10°C

IonCode™ Barcode Adapters 1–384 Kit

The IonCode™ Barcode Adapters 1–384 Kit (Cat. No. A29751) provides 384 different premixed adapters in a convenient 96-well plate format. These barcode adapters, or Ion Xpress™ Barcode Adapters, are required to run multiple libraries per sequencing chip, and are ordered separately.

Component and cap color	Quantity	No. of reactions	Storage
IonCode™ Barcode Adapters 1–384 Kit • IonCode™ 0101–0196 in 96-well PCR Plate (red) • IonCode™ 0201–0296 in 96-well PCR Plate (yellow) • IonCode™ 0301–0396 in 96-well PCR Plate (green) • IonCode™ 0401–0496 in 96-well PCR Plate (blue)	4 × 96-well plates (20 µL/well)	3,840 (10 reactions per barcode)	–30°C to –10°C

Ion Xpress™ Barcode Adapters Kits

Each kit provides 16 different barcode adapters, sufficient for ~640 Ion AmpliSeq™ libraries. These barcode adapters, or IonCode™ Barcode Adapters, are required to run multiple libraries per sequencing chip, and are ordered separately.

Component and cap color	Quantity	Volume per tube	Storage
Ion Xpress™ P1 Adapter (violet)	1 tube	320 µL	–30°C to –10°C
Ion Xpress™ Barcode X (white)	16 tubes (1 per barcode)	20 µL each	

The following Ion Xpress™ Barcode Adapters Kits are available.

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

Ion Library Equalizer™ Kit

The Ion Library Equalizer™ Kit (Cat. No. 4482298) provides a streamlined method for normalizing library concentration at 100 pM without quantification.

The kit contains reagents sufficient for 96 libraries.

Component	Amount	Storage
Equalizer™ Primers (pink cap)	200 µL	2°C to 8°C
Equalizer™ Capture (purple cap)	1 mL	
Equalizer™ Elution Buffer (clear cap)	10 mL	
Equalizer™ Beads (orange cap)	300 µL	
Equalizer™ Wash Buffer (clear cap)	35 mL	15–30°C[1]

[1] Can be stored at 2–8°C.

Ion AmpliSeq™ panels

Ion AmpliSeq™ panels provide pools of primers for the 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. Panels are designed to be used with nucleic acid from various sources, including nucleic acid from formalin-fixed paraffin-embedded (FFPE) tissue and cell-free DNA (cfDNA).

Order Ion AmpliSeq™ panels using Ion AmpliSeq™ Designer. Users who are interested in inherited disease or germline research applications can design and order panels using a content selection engine or by uploading their own gene list. The gene designs in the On-Demand catalog have been optimized for high performance. For additional information, see Appendix D, "Ion AmpliSeq™ panels" or visit **AmpliSeq.com**.

The following types of panels are compatible with the Ion Chef™ System.

Product and description	In stock panels		Made–to–order panels	Fixed panels	
	Oncomine™ tumor specific panels	Ion AmpliSeq™ On-Demand Panels	Ion AmpliSeq™ Custom Panels	Ion AmpliSeq™ Community Panels	Ion AmpliSeq™ Ready-to-Use Panels
Genome	Human	Human	Any	Human	Human
Nucleic Acid	DNA/RNA	DNA only	DNA/RNA	DNA/RNA	DNA/RNA
Sample type	FFPE	Genomic DNA	Any	Any	Any
Target type	Genes	Genes	Genes, regions, hotspots, amplicons, expression, fusions	Genes, regions, hotspots, amplicons, expression, fusions	Genes, regions, hotspots, amplicons, expression, fusions

Recommended materials and equipment

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Item	Source
Ion AmpliSeq™ Sample ID Panel[1]	4479790
Ion AmpliSeq™ panel kits	
Ion AmpliSeq™ Transcriptome Human Gene Expression Kit[2]	A26325
Ion AmpliSeq™ Transcriptome Mouse Gene Expression Kit[3]	A36553
Ion AmpliSeq™ Exome RDY Kits[4]	A38262, A38264

(continued)

Item	Source
Additional equipment	
One of the following Applied Biosystems™ real-time PCR instruments. • 7500 Real-Time PCR System • 7900HT Fast Real-Time PCR System[5] • StepOne™ Real-Time PCR System • StepOnePlus™ Real-Time PCR System • ViiA™ 7 Real-Time PCR System • QuantStudio™ Real-Time PCR Systems	**MLS**
Fisher Scientific™ Mini Plate Spinner Centrifuge	Fisher Scientific™, 14-100-143
MicroAmp™ Adhesive Film Applicator	4333183
Nucleic acid isolation	
Ion AmpliSeq™ Direct FFPE DNA Kit	A31133, A31136
RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	AM1975
RecoverAll™ Multi-Sample RNA/DNA Workflow	A26069
MagMAX™ FFPE Total Nucleic Acid Isolation Kit	4463365
MagMAX™ FFPE DNA/RNA Ultra Kit	A31881
PureLink™ Genomic DNA Mini Kit	K1820-00
Nucleic acid quantification	
TaqMan™ RNase P Detection Reagents Kit *(Recommended for DNA only)*	4316831
Qubit™ 4 Fluorometer[6] with one of these quantitation kits. • Qubit™ dsDNA HS Assay Kit (DNA) • Qubit™ RNA HS Assay Kit (RNA)	Q33238 • Q32851, Q32854 • Q32852, Q32855
Additional material for the Ion AmpliSeq™ Direct FFPE DNA Kit	
(Optional) Invitrogen™ Uracil DNA Glycosylase (UDG) *(Optional)* Thermo Scientific™ Uracil-DNA Glycosylase (1 U/µL) (UDG)	18054015 EN0361
Additional material for purified FFPE DNA	
Thermo Scientific™ Uracil-DNA Glycosylase, heat-labile	78310100UN

(continued)

Item	Source
Controls	
AcroMetrix™ Oncology Hotspot Control	969056
Control DNA (from CEPH Individual 1347-02)	403062

[1] Used to add amplicons that create a unique identifier for human gDNA samples.
[2] See the *Ion AmpliSeq™ Transcriptome Human Gene Expression Kit User Guide* (Pub. No. MAN0010742).
[3] See the *Ion AmpliSeq™ Transcriptome Mouse Gene Expression Kit User Guide* (Pub. No. MAN0017343).
[4] See the *Ion AmpliSeq™ Exome RDY Library Preparation User Guide* (Pub. No. MAN0010084).
[5] Supported but no longer available for purchase.
[6] Qubit™ 2.0 Fluorometer and later are supported.

Oncomine™ panels and assays

The Ion AmpliSeq™ Library Kit Plus is recommended for use with Oncomine™ panels and assays. For more information about available panels and assays, see **https://www.thermofisher.com/us/en/home/clinical/preclinical-companion-diagnostic-development/oncomine-oncology.html**.

3 Before you begin

Procedural guidelines

- Minimize freeze-thaw cycles of panels by aliquoting as needed for your experiments. Panels can be stored at 4°C for 1 year.
- Use good laboratory practices to minimize cross-contamination of products. If possible, perform PCR setup in an area or room that is free of amplicon contamination. Always change pipette tips between samples.
- Use a calibrated thermal cycler specified in “Required materials not supplied” on page 11.
- Pipet viscous solutions, such as 5X Ion AmpliSeq™ HiFi Mix, FuPa Reagent, Switch Solution, DNA Ligase, and panels, slowly and ensure complete mixing by vortexing or pipetting up and down several times.

Tips

- Target amplification reaction master mixes can be made with 5X Ion AmpliSeq™ HiFi Mix and primer pools, transferred to a 96-well plate, and sample DNA added. However, be careful to add equal amounts of DNA to avoid pool imbalance.
- Arrange samples in columns on the plate for easier pipetting with multichannel pipettes during purification with the DynaMag™–96 Side Magnet.
- If you observe evaporation in target amplification reactions, avoid using outside wells.
- Plate seals can be firmly applied using the MicroAmp™ Adhesive Film Applicator. Plate seals can be removed with less effort when hot. Try removing seals right after taking the plate out of the thermal cycler.
- Use Ion Torrent™ Dual Barcode Adapter or IonCode™ Barcode Adapters to avoid handling and diluting adapters. Alternatively, combine and dilute Ion Xpress™ Barcode Adapters in large batches and carefully aliquot into 96-well plates.
- When using a Qubit™ 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 adding a proportionally larger volume to a combined library or template preparation.
- When amplifying multiple samples in a single PCR plate, ensure that the input across the samples is about 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 >4 nM in concentration.
- When setting up sample-specific master mixes for panels with two 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 Eppendorf™ DNA LoBind™ Microcentrifuge Tubes before target amplification.

Before each use of the kit

- Thaw components that contain enzymes—such as 5X Ion AmpliSeq™ HiFi Mix, FuPa Reagent, DNA Ligase, and 1X Library Amp Mix—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 Switch Solution after thawing, vortex or pipet up and down at room temperature to resuspend.
- MagMAX™ Pure Bind Beads stored at room temperature can be used immediately after mixing thoroughly to disperse the beads before use. If using Agencourt™ AMPure™ XP Reagent or MagMAX™ Pure Bind Beads stored at 4°C, bring to room temperature for 30 minutes before use, then mix thoroughly to disperse the beads before use.

IMPORTANT! Do NOT substitute a Dynabeads™-based purification reagent for the MagMAX™ Pure Bind Beads.

4 Prepare Ion AmpliSeq™ DNA libraries

Isolate genomic DNA

Guidelines for DNA isolation and quantification

- See “Recommended materials and equipment” on page 14 for recommended kits for isolating gDNA.
- We recommend that you use 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* (Pub. No. MAN0007732) available at **thermofisher.com**).
- The Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) can also be used for quantification, particularly for FFPE DNA, and highly degraded DNA samples.
- 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, low library yields, and poor chip loading.
- The Ion AmpliSeq™ Direct FFPE DNA Kit bypasses nucleic acid isolation when preparing libraries from FFPE sections on slides. See the *Ion AmpliSeq™ Direct FFPE DNA Kit User Guide* (Pub. No. MAN0014881) for a protocol for using this kit to prepare gDNA from FFPE tissue.
- The Direct FFPE DNA preparation can be stored for up to 6 months at –20°C before library preparation.

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 tissue, FFPE tissue, or cfDNA.
- Increasing the amount of DNA results in higher-quality libraries, especially when DNA quality or quantity is unknown. We recommend that you use 1 ng gDNA (300 copies) only with high-quality, well-quantified samples.
- Ion AmpliSeq™ On-Demand Panels, and some Ion AmpliSeq™ Ready-to-Use Panels, Ion AmpliSeq™ Made-to-Order Panels and Ion AmpliSeq™ Community Panels for DNA, are provided as multiple primer pools to create overlapping amplicons to cover large target regions. Panels with 3 or 4 primer pools require additional DNA and library reagents compared with 1- and 2-primer pool panels.
- The maximum volume of DNA per reaction depends on the concentration of the Ion AmpliSeq™ primer pool that you use, the number of primer pools in the panel, and whether you use a spike-in panel, such as the Ion AmpliSeq™ Sample ID Panel.

  See “Prepare DNA target amplification reactions” on page 20 (1 primer pool and 2 primer pools) and “Prepare DNA target amplification reactions for panels with 3 or 4 primer pools” on page 66 for the maximum volume of DNA in target amplification reactions.
- For Ion AmpliSeq™ Direct FFPE DNA Kit 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.

Input DNA prepared using the Ion AmpliSeq™ Direct FFPE DNA Kit

If you are using DNA prepared using the Ion AmpliSeq™ Direct FFPE DNA Kit, follow the instructions in the *Ion AmpliSeq™ Direct FFPE DNA Kit User Guide* (Pub. No. MAN0014881), then proceed to “Prepare DNA target amplification reactions” on page 20.

Prepare DNA target amplification reactions

Use one of the following procedures to prepare DNA target amplification reactions for Ion AmpliSeq™ DNA panels with 1 or 2 primer pool.

For Ion AmpliSeq™ DNA panels with 3 or 4 primer pools, see “Prepare DNA target amplification reactions for panels with 3 or 4 primer pools” on page 66.

If you use purified FFPE DNA, we recommend that you remove deaminated cytosine (uracil) bases enzymatically by treatment with Uracil DNA Glycosylase (UDG) before the target amplification reaction. For instructions, see “(Optional) Remove deaminated bases from Direct FFPE DNA” on page 72.

Prepare DNA target amplification reactions—1 primer pool

For DNA panels with 1 primer pool, target amplification reactions can be assembled directly in a 96-well plate, one well per sample. Prepare the reactions based on whether you are using a 2X primer pool panel or a 5X primer pool panel. For multiple reactions, prepare a master mix without sample DNA.

If using the Ion AmpliSeq™ Direct FFPE DNA Kit, see *Ion AmpliSeq™ Direct FFPE DNA Kit User Guide* (Pub No. MAN0014881)

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. If using the Ion AmpliSeq™ Direct FFPE DNA Kit, add up to the maximum volume of DNA indicated in the following table to each well to achieve the maximum amount of DNA in the reaction.

   For sample DNA prepared otherwise, add up to 100 ng of DNA to each well.

Component	Volume
2X single-primer pool panel	
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 single-primer pool panel	
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. Add the remaining reagents to the wells in the volumes that are indicated in the table. Bring the total volume to 20 µL.

3. Seal the plate with a MicroAmp™ Clear Adhesive Film.

   To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

4. Place a MicroAmp™ Optical Film Compression Pad on the plate, then load the plate into the thermal cycler.

Proceed to “Amplify the targets” on page 24.

Prepare DNA target amplification reactions—2 primer pools

If you are using a DNA panel with 2 primer pools, set up two 10-µL amplification reactions, then combine them after target amplification to yield a total volume of 20 µL. Prepare the reactions based on whether you are using a 2X primer pool panel or a 5X primer pool panel.

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.

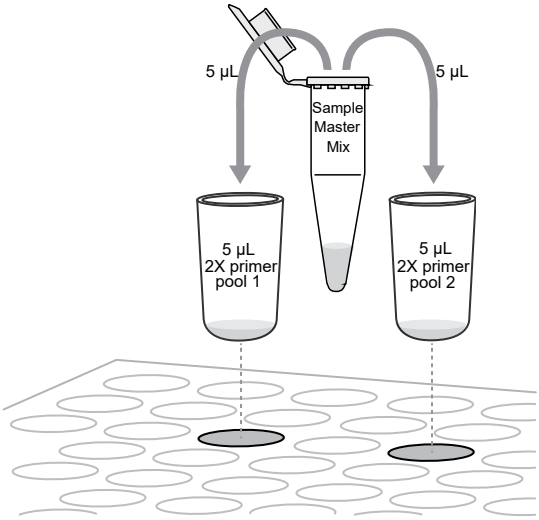
1. For DNA panels with 2 primer pools, use the following table to prepare for each sample a target amplification master mix without primers in a 1.5-mL tube.

   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. Add to the target amplification reaction master mix.

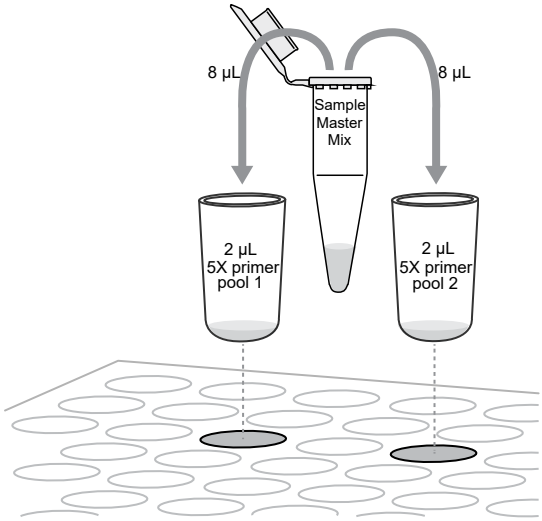
Component	Volume
2X 2-primer pool panel	
5X Ion AmpliSeq™ HiFi Mix (red cap)	5 µL
DNA (2–100 ng), or Direct FFPE DNA preparation	≤7.5 µL
Nuclease-free water	to 12.5 µL
5X 2-primer pool panel	
5X Ion AmpliSeq™ HiFi Mix (red cap)	4.5 µL
DNA (2–100 ng), or Direct FFPE DNA preparation	≤13.5 µL
Nuclease-free water	to 18 µL

2. Mix thoroughly by pipetting up and down 5 times, then transfer sample-specific master mixes to 2 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.

2-pool panels at 2X concentration



2-pool panels at 5X concentration



   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™ Clear Adhesive Film.

   To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

4. Place a MicroAmp™ Optical Film Compression Pad on the plate, then load the plate into the thermal cycler.

Proceed to “Amplify the targets”.

Amplify the targets

1. To amplify target regions, run the following program.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	99°C	2 minutes
Cycle Set the cycle number according to the following tables.	Denature	99°C	15 seconds
	Anneal and extend	60°C	4/8/16 minutes Set the time according to the following tables.
Hold	—	10°C	Hold

The following amplification cycle parameters are recommended based on 10-ng DNA input.

Primer pairs per pool	Recommended number of amplification cycles (10 ng DNA, 3,000 copies)		Anneal/extend time
	High quality DNA	Low quality DNA (FFPE DNA or cfDNA)	
12–24	21	24	4 minutes
25–48	20	23	4 minutes
49–96	19	22	4 minutes
97–192	18	21	4 minutes
193–384	17	20	4 minutes
385–768	16	19	4 minutes
769–1,536	15	18	8 minutes
1,537–3,072	14	17	8 minutes
3,073–6,144	13	16	16 minutes
6,145–24,576	12	15	16 minutes

Exceptions can be made to the recommended parameters for the following panels.

Ion AmpliSeq™ panel	Primer pairs per pool	Description of change
Ion AmpliSeq™ Comprehensive Cancer Panel (Cat. No. 4477685)	~4,000	Use 8 minutes anneal/extend time instead of 16 minutes
Ion AmpliSeq™ panels using a 375-bp amplicon design	—	Add 4 minutes to the anneal/extend time recommended in the table

2. *(Optional)* Adjust the cycle number from the preceding table for lower or higher DNA input.

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

Note:

- The cycle number can be increased when input material quality or quantity is questionable.
- If 2 primer pools for a single panel fall into different cycling categories, use the greater number of cycles.
- When amplifying multiple samples in a single PCR plate, ensure that the input across the samples is about equivalent. In this way, 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 (only for DNA libraries with 2 primer pools)

1. Tap the plate gently on a hard flat surface, or briefly centrifuge to collect the contents.
2. Carefully remove the plate seal.
3. For each sample, combine the 10-µL target amplification reactions. The total volume for each sample should be 20 µL.

Proceed to step 2 of “Partially digest amplicons”.

Partially digest amplicons

IMPORTANT! FuPa Reagent is viscous. Pipet slowly and mix thoroughly. Perform this step on ice or on a cold block, then quickly proceed to incubation.

1. For one primer pool panel, tap the plate gently on a hard flat surface, or briefly centrifuge to collect the contents, then remove the plate seal.
2. Add 2 µL of FuPa Reagent (brown cap) to each amplified sample. The total volume is ~22 µL.
3. Seal the plate with a MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

4. Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate 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.

5. Tap the plate gently on a hard flat surface, or briefly centrifuge to collect the contents.

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

Ligate adapters to the amplicons and purify

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

Ion Torrent™ Dual Barcode Adapters are provided at the appropriate concentration. No further handling is necessary.

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

Ion Xpress™ Barcode Adapters require handling and dilution as described in this user guide.

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

Combine and dilute adapters (only for Ion Xpress™ Barcode Adapters)

For each barcode X that is selected, prepare a mix of Ion P1 Adapter and Ion Xpress™ barcode X at a final dilution of 1:4 for each adapter. Scale volumes as necessary. Use 2 µL of this barcode adapter mix in step 4 in “Perform the ligation reaction”.

For example, combine the volumes that are listed in the following table.

Component	Volume
Ion P1 Adapter	2 µL
Ion Xpress™ barcode X, where X is the chosen barcode	2 µL
Nuclease-free water	4 µL
Total	**8 µL**

Store diluted adapters at –20°C.

Perform the ligation reaction

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

2. Briefly centrifuge the plate to collect the contents.

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

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

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

4. Seal the plate with a new MicroAmp™ Clear 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 5 times before sealing the plate.

5. Place a MicroAmp™ Optical Film 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 for up to 24 hours at 10°C on the thermal cycler. For longer periods, store at –20°C.

Purify the library

IMPORTANT!

- The recommended product for purification is MagMAX™ Pure Bind Beads. Another option is Agencourt™ AMPure™ XP Reagent.
- MagMAX™ Pure Bind Beads stored at room temperature can be used immediately after mixing thoroughly to disperse the beads before use. If using Agencourt™ AMPure™ XP Reagent or MagMAX™ Pure Bind Beads stored at 4°C, bring to room temperature for 30 minutes before use, then mix thoroughly to disperse the beads before use.
- Pipet the solution slowly.
- Do NOT substitute a Dynabeads™-based purification reagent for the MagMAX™ Pure Bind Beads.

1. Briefly centrifuge the plate to collect the contents.
2. Carefully remove the plate seal, then add 45 µL (1.5X sample volume) of MagMAX™ Pure Bind Beads to each library. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.

   Visually inspect each well to ensure that the mixture is homogeneous.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the 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.
5. 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. Carefully remove, then discard the supernatant without disturbing the pellet.

   If your magnet does not have two positions for shifting the beads, remove the plate from the magnet and gently pipet up and down 5 times (with the pipettor set at 100 µL). Return the plate to the magnet and incubate for 2 minutes or until the solution clears.
6. Repeat step 5 for a second wash.
7. 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 to collect the contents, then remove any remaining ethanol before air-drying the beads. Under conditions of low relative humidity, the beads air-dry rapidly. Do not overdry.

Proceed to library equalization or quantification

Proceed to one of the following:

- Option 1: Equalize the library (see Chapter 6, “Equalize the library”).
- Option 2: Quantify the library by qPCR (see Chapter 7, “Quantify the library by qPCR”).
- Option 3: Quantify the amplified library with a Qubit™ Fluorometer, or with the Agilent™ 2100 Bioanalyzer™ Instrument. See Chapter 8, “Quantify the amplified library with a Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ Instrument”).

5 Prepare Ion AmpliSeq™ RNA libraries

Guidelines for RNA isolation, quantification, and input

- See "Recommended materials and equipment" on page 14 for kits recommended for isolating total RNA.
- We recommend that you use 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 usually results in higher quality libraries, especially when RNA quality or quantity is unknown. We recommend that you use 1 ng total RNA only with high-quality, well-quantified samples.

Reverse transcribe the RNA

If you are starting from RNA, you must first reverse transcribe the RNA to cDNA. We recommend that you use one of the following kits.

- SuperScript™ IV VILO™ Master Mix (Cat. No. 11756050; ordered separately).
- Ion Torrent™ NGS Reverse Transcription Kit (Cat. No. A45003; ordered separately). Using this kit simplifies reaction setup and provides improved performance

If you are using the SuperScript™ VILO™ cDNA Synthesis Kit (Cat. No. 11754050) , see "Reverse transcribe RNA with the SuperScript™ VILO™ cDNA Synthesis Kit" on page 71 for setup instructions.

1. If the 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.

Component	Volume	
	VILO	NGS
SuperScript™ IV VILO™ Master Mix	2 µL	—
NGS 5X Reaction Buffer	—	2 µL
NGS 10X RT Enzyme Mix	—	1 µL
Total RNA (1–100 ng)	≤8 µL	≤7 µL
Nuclease-free water	to 10 µL	to 10 µL
Total volume per well	**10 µL**	**10 µL**

3. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.

   Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

4. Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate in the thermal cycler, then run the following program to synthesize cDNA.

Temperature	Time
25°C	10 minutes
50°C	10 minutes
85°C	5 minutes
10°C	Hold

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

5. Gently tap the plate on the bench to ensure that reactions are at the bottom of the wells, or if possible, centrifuge the plate to collect the contents. Proceed to the next step.

   - If you are using an RNA panel with 1 primer pool, proceed to “Prepare cDNA target amplification reactions—1 primer pool” on page 32.
   - If you are using an RNA panel with 2 primer pools, proceed to “Prepare cDNA target amplification reactions—2 primer pools”.

Prepare cDNA target amplification reactions—1 primer pool

For RNA panels with 1 primer pool, target amplification reactions can be assembled directly in a 96-well plate.

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 RNA panels with 1 primer pool, remove the seal from the plate, then 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™ Clear Adhesive Film, place a MicroAmp™ Optical Film Compression Pad on the plate, then place the sealed plate in a thermal cycler .

   To prevent evaporation during target amplification, use the applicator tool that is supplied with the film to press the film securely around each reaction well and around the perimeter of the plate.

Proceed to “Amplify the targets” on page 33.

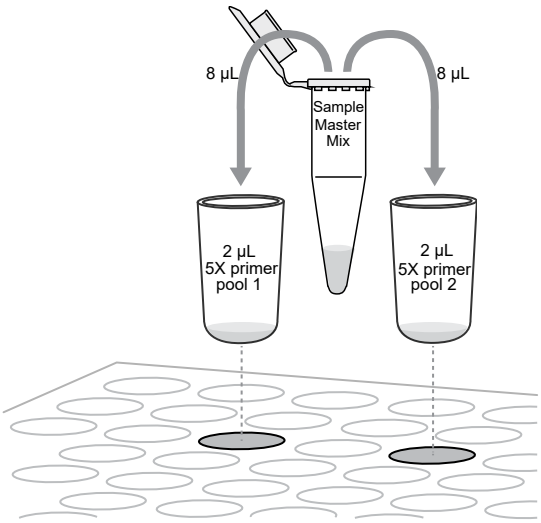
Prepare cDNA target amplification reactions—2 primer pools

If you are using an RNA panel with 2 primer pools, set up two 10-µL target amplification reaction volumes, then combined them after target amplification to yield a total volume of 20 µL.

1. For RNA panels with 2 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 5 times, then transfer 8 µL of each sample-specific master mix 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 for a total of 10 µL in each well.

4. Seal the plate with a MicroAmp™ Clear Adhesive Film, place a MicroAmp™ Optical Film Compression Pad on the plate, then place the sealed plate in a thermal cycler.

   To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

Proceed to “Amplify the targets” on page 33.

Amplify the targets

1. To amplify target regions, run the following program.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	98°C	2 minutes
Cycle Set the number according to the following tables.	Denature	98°C	15 seconds
	Anneal and extend	60°C	4/8/16 minutes Set the time according to the following tables.
Hold	—	10°C	Hold

   The following amplification cycle parameters are recommended based on 10-ng DNA input.

   When using RNA panels with a mix of primer pairs for gene expression and gene fusion detection, use the number of gene expression primer pairs only to determine the appropriate number of amplification cycles.

Primer pairs per pool (excluding gene fusion primer pairs)	Recommended number of amplification cycles (10 ng RNA)		Anneal/extend time
	High quality RNA	Low quality RNA (FFPE RNA or cfRNA)	
Panels for gene fusion detection only	27	30	4 minutes
12–24	21	24	4 minutes
25–48	20	23	4 minutes
49–96	19	22	4 minutes
97–192	18	21	4 minutes
193–384	17	20	4 minutes
385–768	16	19	4 minutes
769–1,536	15	18	8 minutes
1,537–3,072	14	17	8 minutes
3,073–6,144	13	16	16 minutes
6,145–24,576	12	15	16 minutes

2. (Optional) Adjust the cycle number from the preceding table for lower or higher RNA input.

Amount of RNA starting material	Adjustment to cycle number
1 ng	+3
10 ng	0
100 ng	–3

Note:

- The cycle number can be increased when input material quality or quantity is questionable.
- If 2 primer pools for a single panel fall into different cycling categories, use the greater number of cycles.
- When amplifying multiple samples in a single PCR plate, ensure that the input across the samples is about 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 (only for RNA libraries with 2 primer pools)

1. Tap the plate gently on a hard flat surface, or briefly centrifuge to collect the contents.
2. Carefully remove the plate seal.
3. For each sample, combine the 10-µL target amplification reactions. The total volume for each sample should be 20 µL.

Proceed to step 2 of "Partially digest amplicons".

Partially digest amplicons

IMPORTANT! FuPa Reagent is viscous. Pipet slowly and mix thoroughly. Perform this step on ice or on a cold block, then quickly proceed to incubation.

1. For one primer pool panel, tap the plate gently on a hard flat surface, or briefly centrifuge to collect the contents, then remove the plate seal.
2. Add 2 µL of FuPa Reagent (brown cap) to each amplified sample. The total volume is ~22 µL.
3. Seal the plate with a MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
4. Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate 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.

5. Tap the plate gently on a hard flat surface, or briefly centrifuge to collect the contents.

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

Ligate adapters to the amplicons and purify

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

Ion Torrent™ Dual Barcode Adapters are provided at the appropriate concentration. No further handling is necessary.

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

Ion Xpress™ Barcode Adapters require handling and dilution as described in this user guide.

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

Combine and dilute adapters (only for Ion Xpress™ Barcode Adapters)

For each barcode X that is selected, prepare a mix of Ion P1 Adapter and Ion Xpress™ barcode X at a final dilution of 1:4 for each adapter. Scale volumes as necessary. Use 2 µL of this barcode adapter mix in step 4 in “Perform the ligation reaction”.

For example, combine the volumes that are listed in the following table.

Component	Volume
Ion P1 Adapter	2 µL
Ion Xpress™ barcode X, where X is the chosen barcode	2 µL
Nuclease-free water	4 µL
Total	**8 µL**

Store diluted adapters at –20°C.

Perform the ligation reaction

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

2. Briefly centrifuge the plate to collect the contents.

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

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

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

4. Seal the plate with a new MicroAmp™ Clear 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 5 times before sealing the plate.

5. Place a MicroAmp™ Optical Film 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 for up to 24 hours at 10°C on the thermal cycler. For longer periods, store at –20°C.

Purify the library

IMPORTANT!

- The recommended product for purification is MagMAX™ Pure Bind Beads. Another option is Agencourt™ AMPure™ XP Reagent.
- MagMAX™ Pure Bind Beads stored at room temperature can be used immediately after mixing thoroughly to disperse the beads before use. If using Agencourt™ AMPure™ XP Reagent or MagMAX™ Pure Bind Beads stored at 4°C, bring to room temperature for 30 minutes before use, then mix thoroughly to disperse the beads before use.
- Pipet the solution slowly.
- Do NOT substitute a Dynabeads™-based purification reagent for the MagMAX™ Pure Bind Beads.

1. If possible, briefly centrifuge the plate to collect the contents.
2. Carefully remove the plate seal, then add 45 µL (1.5X sample volume) of MagMAX™ Pure Bind Beads to each library. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.

   Visually inspect each well to ensure that the mixture is homogeneous.
3. Incubate the mixture for 5 minutes at room temperature.
4. 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.
5. 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. Carefully remove, then discard the supernatant without disturbing the pellet.

   If your magnet does not have two positions for shifting the beads, remove the plate from the magnet and gently pipet up and down 5 times (with the pipettor set at 100 µL). Return the plate to the magnet and incubate for 2 minutes or until the solution clears.
6. Repeat step 5 for a second wash.
7. Ensure that all ethanol droplets are removed from the wells. Keep the plate in the magnet, then air-dry the beads at room temperature for 1–5 minutes. Do not overdry.

   IMPORTANT! Residual ethanol drops inhibit library amplification. If needed, centrifuge the plate to collect the contents, then remove remaining ethanol before air-drying the beads. Under conditions of low relative humidity the beads air-dry rapidly. Do not overdry.

Proceed to library equalization or quantification

Proceed to one of the following:

- Option 1: Equalize the library (see Chapter 6, “Equalize the library”).
- Option 2: Quantify the library by qPCR (see Chapter 7, “Quantify the library by qPCR”).
- Option 3: Quantify the amplified library with a Qubit™ Fluorometer, or with the Agilent™ 2100 Bioanalyzer™ Instrument. See Chapter 8, “Quantify the amplified library with a Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ Instrument”).

6 Equalize the library

IMPORTANT! Equalize the library using one of these methods.

- Quantitative PCR (qPCR) is the most simple and robust method to equalize the library, and is recommended for libraries from RNA and samples of unknown quality or quantity.

  We recommend that you start with the qPCR library quantification method (see Chapter 7, "Quantify the library by qPCR").
- When library yields are consistently above the minimum concentrations that are stated in this user guide, the Ion Library Equalizer™ Kit can be used reliably to equalize the library. This method offers the greatest convenience, but can result in a higher proportion of libraries with low yield and read number when sample quality or quantity is low.

  This method normalizes 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, libraries that are run on the same chip can be combined during the equalization process to improve balance and reduce hands-on time.

  Standard library amplification parameters are not compatible with the Ion Library Equalizer™ Kit.
- Alternatively, Qubit™ Fluorometer and Agilent™ 2100 Bioanalyzer™ Instrument methods can be used to equalize the library (see Chapter 8, "Quantify the amplified library with a Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ Instrument").

  Qubit™ Fluorometer use is the most economical, but lacks specificity. Agilent™ 2100 Bioanalyzer™ Instrument assessment generates the most information for troubleshooting.

If sample quality or quantity is variable or unknown (such as RNA from FFPE tissue, or Direct FFPE DNA), the qPCR and Qubit™ Fluorometer and Agilent™ 2100 Bioanalyzer™ Instrument quantification methods may provide a higher success rate in terms of library yield and resulting number of sequencing reads.

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 with purified libraries from the plate magnet, then add 50 µL of 1X Library Amp Mix (black cap) and 2 µL of Equalizer™ Primers (pink cap), or 2 µL of 25X Library Amp Primers, to each bead pellet. The 1X Library Amp Mix and primers can be combined before addition.

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 5 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, then 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)

5. (Optional) If possible after thermal cycling, briefly centrifuge the plate to collect the contents.

   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 be used only when library concentrations are >4,000 pM after library amplification.

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

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

   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, wash the beads again with an equal volume of Equalizer™ Wash Buffer.

2. For each reaction, pipet 3 µL of beads into a clean 1.5-mL tube, then add 6 µL per reaction of Equalizer™ Wash Buffer.

   For example, if you have 4 reactions, add 12 µL of beads and 24 µL of Equalizer™ Wash Buffer.

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

4. Carefully remove the supernatant without disturbing the pellet, then discard.

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

Add Equalizer™ Capture to the amplified library

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

   The final equalized library concentration depends on accurate pipetting of the Equalizer™ Capture reagent.

2. Seal the plate with a MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.

   Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

3. 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 79 or “Ion Chip™ capacities for Ion AmpliSeq™ RNA libraries” on page 80.

2. Carefully remove the seal from the plate, then 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 are to 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 are to 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.

   Save uncombined individual libraries for repeat analysis, if needed.

Add Equalizer™ Beads and wash

1. Gently vortex or pipet up and down to mix the washed Equalizer™ Beads.

2. If needed, carefully remove the seal from the plate, then add 6 µL of washed Equalizer™ Beads to each plate well that contains the captured library (either combined or individual).

3. Set the pipette volume to 40 µL, then pipet the mixture up and down at least 5 times to mix thoroughly.

4. Incubate at room temperature for 5 minutes.

   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 the contents.

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

6. If needed, carefully remove the seal from the plate, then remove the supernatant without disturbing the pellet.

   Save the supernatant for repeat analysis, if needed.

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

8. To wash the beads, move the plate side-to-side in the two positions of the magnet.

   If your magnet does not have two positions for shifting the beads, remove the plate from the magnet, set a pipettor to at least half the total volume, then gently pipet the contents up and down 5 times. 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 step 7 through step 9.

    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™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

   Centrifuge with enough force to collect the contents, but not the 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 in “Store libraries” on page 44.

(Optional) Combine equalized libraries

Combine the equalized libraries based on the number of libraries to be run on a single chip, as calculated in Appendix C, “Strategies for combining Ion AmpliSeq™ libraries”.

Store libraries

Store libraries at 4–8°C for up to 1 month. For longer lengths of time, store at –30°C to –10°C.

Note: We recommend transferring the supernatant to a 1.5-mL Eppendorf™ LoBind™ tube for long-term storage.

7 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 and dilute the library

1. Remove the plate with purified libraries from the plate magnet, then add 50 µL of Low TE to the pellet to disperse the beads.
2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.

   Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
3. Incubate at room temperature for at least 2 minutes.
4. Place the plate on the magnet for at least 2 minutes.
5. Prepare a 100-fold dilution for quantification. Remove 2 µL of supernatant that contains the library, then combine with 198 µL of nuclease-free water.

Proceed immediately to “Quantify library by qPCR and calculate the dilution factor” on page 46.

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 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 Ion Library qPCR Master Mix and 2 µL of Ion Library TaqMan™ Quantitation Assay, 20X , 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. Seal the plate with a MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.

5. 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 qPCR Master Mix can be used on various instruments, as listed in the following table. The fast cycling program was developed using the StepOnePlus™ Real-Time PCR 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	Stage	Temperature	Time
Fast run mode				
7500 Fast	96-well Fast	Hold (UDG incubation)	50°C	2 minutes
7900 HT 7900 HT Fast	96-well Fast 384-well Standard	Hold (polymerase activation)	95°C	20 seconds
ViiA™ 7		Cycle (40 cycles)	95°C	1 second
QuantStudio™ 3, 5, or 7				
StepOne™ StepOnePlus™	48-/96-well Fast		60°C	20 seconds
Standard run mode				
7300	96-well Standard	Hold (UDG incubation)	50°C	2 minutes
7500		Hold (polymerase activation)	95°C	2 minutes
7900 HT 7900 HT Fast		Cycle (40 cycles)	95°C	15 seconds
ViiA™ 7			60°C	1 minute
QuantStudio™ 3, 5, or 7				

6. After qPCR, calculate the average concentration of the undiluted Ion AmpliSeq™ library by multiplying the concentration that is determined with qPCR by 100.

7. Based on the calculated library concentration, determine the dilution that results in a concentration of ~50–100 pM.
   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.

8. Dilute the library to ~50–100 pM, combine, then proceed to template preparation, or store libraries as described below.

(Optional) Combine amplicon libraries

Multiple strategies for combining Ion AmpliSeq™ libraries are available. See Appendix C, “Strategies for combining Ion AmpliSeq™ libraries”.

Proceed to templating and sequencing

Proceed to template preparation and sequencing, or store libraries as descriped in “Store libraries” on page 44.

Store libraries

Store libraries at 4–8°C for up to 1 month. For longer lengths of time, store at –30°C to –10°C.

Note: We recommend transferring the supernatant to a 1.5-mL Eppendorf™ LoBind™ tube for long-term storage.

8 Quantify the amplified library with a 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 1X Library Amp Mix, then purify. Quantify the library using a Qubit™ 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 unamplified libraries.

Amplify the library

1. Remove the plate with purified libraries from the plate magnet, then add 50 µL of 1X Library Amp Mix and 2 µL of 25X Library Amp Primers to each bead pellet.
   - The 1X Library Amp Mix is used to elute the libraries from the beads.
   - The 1X Library Amp Mix and 25X Library Amp Primers can be combined before addition.
2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.

   Alternatively, mix by pipetting at least half the total volume up and down at least 5 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 MicroAmp™ Clear Adhesive Film, place a MicroAmp™ Optical Film 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 MagMAX™ Pure Bind Beads.

- 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!

- The recommended product for purification is MagMAX™ Pure Bind Beads. Another option is Agencourt™ AMPure™ XP Reagent.
- MagMAX™ Pure Bind Beads stored at room temperature can be used immediately after mixing thoroughly to disperse the beads before use. If using Agencourt™ AMPure™ XP Reagent or MagMAX™ Pure Bind Beads stored at 4°C, bring to room temperature for 30 minutes before use, then mix thoroughly to disperse the beads before use.
- Pipet the solution slowly.
- Do NOT substitute a Dynabeads™-based purification reagent for the MagMAX™ Pure Bind Beads.

First-round purification

1. Tap the plate gently on a hard flat surface, or briefly centrifuge to collect the contents, then remove the plate seal.
2. Add 25 µL (0.5X sample volume) of MagMAX™ Pure Bind Beads to each plate well containing ~50 µL of sample. Mix the bead suspension with the DNA thoroughly by pipetting up and down 5 times.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a magnet, such as the DynaMag™–96 Side Magnet, for at least 5 minutes, or until the solution is clear.

5. 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 in "First-round purification" on page 50, add 60 µL (1.2X original sample volume) of MagMAX™ Pure Bind Beads. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.
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, then discard the supernatant without disturbing the pellet.

   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). 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 2–5 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™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.

   Alternatively, mix by setting a pipettor to 40 µL, then pipet the mixture up and down at least 5 times before sealing the plate.
9. Incubate at room temperature for at least 2 minutes.
10. Place the plate in the magnet for at least 2 minutes, then analyze an aliquot of the supernatant as described in:
    - "Qubit™ Fluorometer—Quantify the library and calculate the dilution factor" on page 52 or
    - "Agilent™ 2100 Bioanalyzer™ Instrument—Quantify the library and calculate the dilution factor" on page 53.

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 a Qubit™ 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™ Fluorometer.
   e. *(Qubit™ 2.0 Fluorometer only)* Calculate the concentration of the undiluted library by multiplying by 20. Alternatively, use the **Calculate Stock Conc.** feature on your instrument.
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 (average 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 that is mixed with 290 µL of Low TE (1:30 dilution) yields approximately 15 ng/mL (~100 pM).
3. Dilute library to ~100 pM, combine, then proceed to template preparation, or store libraries as described in “Store libraries” on page 44.

Agilent™ 2100 Bioanalyzer™ Instrument—Quantify the library and calculate the dilution factor

Analyze 1 µL of amplified library on the Agilent™ 2100 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 instruction of the manufacturer 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.
      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 the library to ~100 pM, combine, then proceed to template preparation, or store libraries as described in “Store libraries” on page 44.

(Optional) Combine amplicon libraries

Multiple strategies for combining Ion AmpliSeq™ libraries are available. See Appendix C, “Strategies for combining Ion AmpliSeq™ libraries”.

Proceed to templating and sequencing

Proceed to template preparation and sequencing, or store libraries as descriped in “Store libraries” on page 44.

Store libraries

Store libraries at 4–8°C for up to 1 month. For longer lengths of time, store at –30°C to –10°C.

Note: We recommend transferring the supernatant to a 1.5-mL Eppendorf™ LoBind™ tube for long-term storage.

A

Troubleshooting

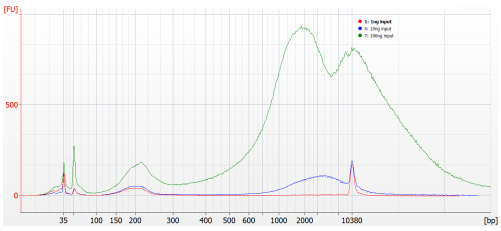
Ion AmpliSeq™ Direct FFPE DNA Kit

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 pipette 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 a scalpel 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, the sample may still be cloudy, this does not affect performance. Ensure homogenous mixing of the sample before removing an aliquot for target amplification. Undissolved tissue that can be aspirated with a pipette 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 the sample.	Use a scalpel blade to scrape away undesired paraffin before 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 the slide in 100% xylene for 30 seconds. 2. Remove the slide, then drain any excess xylene. 3. Submerge the slide in 100% ethanol for 30 seconds. 4. Remove the slide, then allow to air dry.
Difficulty transferring lower (aqueous) phase to target amplification reaction	Transfer Solution is in pipette tip.	Return tip contents to reaction tube, then centrifuge at ≥1,000 × *g* for 1 minute to separate phases. Move pipette quickly through the upper phase when transferring. Transfer Solution does not interfere with target amplification.
Low Ion AmpliSeq™ library concentration **Details:** See "Library yield and quantification" on page 57 for more low library yield troubleshooting.	Insufficient tissue was used.	Use 25–100 mm^2 tissue section of 5–10 µm thickness. If needed, 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 Ion AmpliSeq™ library. Prepare again the FFPE DNA from 100 mm^2 tissue section of 5–10 µm thickness. If needed, 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 concentration (>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 concentration (<0.5 ng/µL) **Details:** Samples with low DNA yield can still be sufficient to generate an Ion AmpliSeq™ library.	FFPE tissue has low DNA content.	Increase the number of target amplification cycles by 2 or 3.

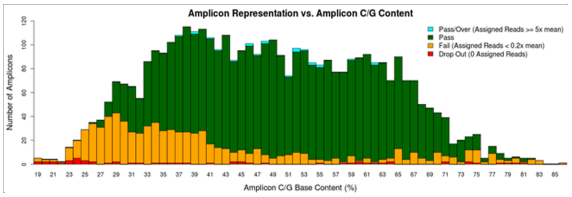
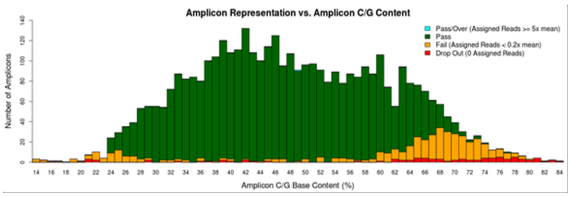
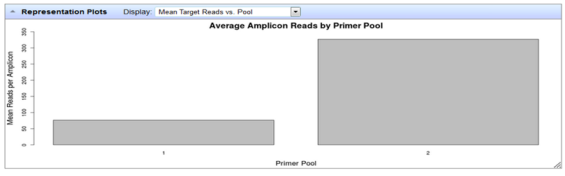
Library yield and quantification

Observation	Possible cause	Recommended action
Library concentration is low—general **Details:** Library concentration is NOT indicative of quality.	Sample DNA or RNA was misquantified.	Requantify sample DNA using the TaqMan™ RNase P Detection Reagents Kit; quantify RNA with a Qubit™ Fluorometer.
	Residual ethanol in sample DNA or RNA inhibited target amplification.	Incubate the uncapped tube in hood for 1 hour.
		Speed-vac the tube at room temperature for 5 minutes.
	Residual ethanol from MagMAX™ Pure Bind Beads purification inhibited library amplification.	Carefully remove all drops of ethanol before library amplification, then centrifuge the plate, if needed.
	Sample DNA or RNA quality was low.	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 amplified library.	Ensure to save the supernatant during first-round purification, and save the bead pellet during the second round.
	MagMAX™ Pure Bind Beads were over-dried.	Do not dry the MagMAX™ Pure Bind Beads more than 5 minutes.
	MagMAX™ Pure Bind Beads inhibited library amplification.	Transfer library off of beads before amplification.
	qPCR cycling time is too short.	Use standard qPCR cycling for library designs >175 bp instead of Fast cycling.
	FFPE RNA was not heat treated before reverse transcription.	Heat FFPE RNA at 80°C for 10 minutes, then cool to room temperature before reverse transcribing.
Library concentration with the Ion Library Equalizer™ Kit is less than expected	Quantity of library before 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.

Observation	Possible cause	Recommended action
Library concentration with the Ion Library Equalizer™ Kit is less than expected *(continued)*	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 concentration is too high	Sample DNA or RNA was mis-quantified.	Requantify sample DNA using the TaqMan™ RNase P Detection Reagents Kit; quantify RNA with a Qubit™ Fluorometer.
	More than 100 ng of sample DNA/RNA was used.	Add less DNA/RNA, or decrease target amplification cycles.
Library concentration is high as measured on the Agilent™ 2100 Bioanalyzer™ Instrument	Markers are misassigned.	Ensure that markers are assigned correctly.
High molecular weight material is present on the Agilent™ 2100 Bioanalyzer™ Instrument or library concentration 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 MagMAX™ Pure Bind Beads 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 or samples with a Qubit™ Fluorometer.
		Reduce target amplification cycle number.

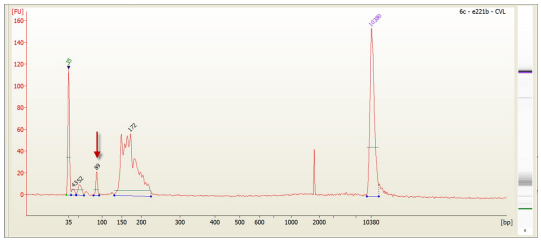
Low amplicon uniformity (DNA only)

Observation	Possible cause	Recommended action
Short amplicons are under-represented	Purification was poor.	Vortex MagMAX™ Pure Bind Beads thoroughly before use, and be sure to dispense the full volume.
		100% ethanol is difficult to pipet accurately; it is essential to prewet pipette tips.
		In post-ligation library purification, increase MagMAX™ Pure Bind Beads volume from 45 µL (1.5X) to 50 µL (1.7X).
		In amplified library purification, increase MagMAX™ Pure Bind Beads 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 (short library reads)	Sample DNA or RNA was 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. **Note:** Use 550 flows as a default setting when sequencing libraries prepared from most Ion AmpliSeq™ On-Demand Panels. In rare instances, amplicons can be longer than 325 bp and can require 650 flows to achieve end-to-end reads, if needed. Determine amplicon length from the panel BED file. Use the templating and sequencing paramaters for 200 bp.
	Sample was over-treated with FuPa Reagent.	Add no more than 2 µL FuPa Reagent per 20 µL target amplification reaction.
		Keep the plate on ice during FuPa Reagent addition, then transfer to a preheated thermal cycler immediately.
	Denaturation temperature was too high.	Use a 97°C enzyme activation/denaturation temperature instead of 99°C in target amplification reactions.

Observation	Possible cause	Recommended action
AT-rich amplicons are under-represented  **Example of loss of AT-rich amplicons.** Within the coverageAnalysis 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.
		Decrease the activate the enzyme and denature temperatures of the target amplification reaction from 99°C to 98°C.
	Digested amplicons were denatured.	Do not exceed 60°C during the amplicon digestion step.
	Digestion was inefficient.	Increase amplicon digestion times to 20 minutes for each step.
GC-rich amplicons are under-represented  **Example of loss of GC-rich amplicons.** Within the coverageAnalysis 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).
	Sample was over-treated with FuPa Reagent.	Add no more than 2 µL FuPa Reagent per 20 µL target amplification reaction.
		Keep the plate on ice during FuPa Reagent addition, then transfer to thermal cycler immediately.
Pool representation is not balanced **Details: Example of pool imbalance**—Within the coverageAnalysis plugin, mean read depth per primer pool is plotted for a 2-pool Ion AmpliSeq™ Panel. In this example, Primer Pool 1 has approximately one quarter the reads of Primer Pool 2.  	Amount of DNA in target amplification reactions varied.	Make a master mix for each sample DNA.
	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.
	Pipetting is inaccurate when pools are combined after target amplification.	Centrifuge the plate after target amplification. Ensure that the entire volume of each pool is removed and combined into a single pool.

Observation	Possible cause	Recommended action
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  **Adapter dimers.** Barcode adapters run at ≈53 bp, and barcode adapter dimers run at ≈105 bp.	Purification was inefficient.	In unamplified library purification, decrease MagMAX™ Pure Bind Beads volume from 45 µL (1.5X) to 30 µL (1X).
		In amplified library purification, decrease MagMAX™ Pure Bind Beads 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 before 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 or 64°C for the first two cycles of the target amplification reaction.
		Lower the DNA input.
	Sample ID Panel targets were 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.
Percentage of polyclonal ISPs is high (>40%)	Library input was too high.	Decrease amount of library added to the template preparation reaction by 50%.

Observation	Possible cause	Recommended action
Percentage of polyclonal ISPs is high (>40%) *(continued)*	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

Supplemental procedures

Modifications to the standard workflow

The following modifications to the standard protocol are designed to allow advanced users to modify and customize the standard Ion AmpliSeq™ protocol successfully.

IMPORTANT! These modifications are unsupported and sometimes can decrease performance.

Shortcuts

- Libraries can be created directly from whole blood that is 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 MagMAX™ Pure Bind Beads. However, any remaining ethanol inhibits the library amplification reaction.
- When using qPCR quantification, the purification step after adapter ligation (“Purify the library”) can be eliminated. Two additional target amplification cycles help reduce the amount of relative by-products that can go into template preparation.
- When using the Agilent™ 2100 Bioanalyzer™ Instrument for quantification (but not a Qubit™ 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 library”). High molecular weight material does not interfere with sequencing, but ensure that the markers are assigned correctly.

- When using the Ion AmpliSeq™ Comprehensive Cancer Panel, the 4 primer pools that are provided can be collapsed into 2 primer pools for library preparation. Use the following procedure.
  a. Mix equal volumes of Pool 1 and Pool 2 (new Pool 1).
  b. Mix equal volumes of Pool 3 and Pool 4 (new Pool 2).
  c. Proceed to "Prepare DNA target amplification reactions—2 primer pools" on page 22. Adjust target amplification cycle number and anneal/extend time for the 2 new pools (~8,000 primer pairs per pool).

  This method produces twice as many libraries from the primer pools, but 2–3% of amplicons can be compromised due to overlap.

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.
- DNA 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 (from 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 20).
  b. Incubate for 5 minutes at room temperature to lyse cells.
  c. Add 1 µL of Single Cell Stop Solution (from the Single 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.

Expand any Ion AmpliSeq™ panel with a spike-in panel

You can modify or expand an Ion AmpliSeq™ On-Demand Panel by adding a spike-in panel. Spike-in panels added to a Ion AmpliSeq™ panel add new content, control assays, and improve coverage uniformity (for example, by adding the same primer pair to 2 or more primer pools).

1. If needed, prepare a 50x (2500 nM) spike-in panel from individual primer pairs using the procedure in "Ion AmpliSeq™ Made-to-Order Panels" on page 83.

2. Add the spike-in panel (at 2,500 nM) to a primary Ion AmpliSeq™ panel using the following guidelines.

Number of primer pairs per pool			Target concentration (combined)	Instructions
Primary panel	Spike-in	Combined		
Chef-Ready primary panels				
Add 6.4 µL of the spike-in panel to the Chef-Ready panel tube.				
All other primary panels				
Less than 96	Up to 123	Less than 96	2X:400 nM	Add 16 µL of the spike-in panel per 100 µL of the primary panel
Less than 96	Up to 123	More than 96	2X:100 nM	Dilute parent panel from 400 nM to 100 nM, then add 4 µL spike-in panel per 100 µL of primary panel
Greater than 96	Up to 123	More than 96	2X:100 nM	Add 4 µL spike-in panel per 100 µL primary panel

3. Mix thoroughly by vortexing, then briefly centrifuge to collect the contents.
   The modified primer pools are ready to use. No adjustment of volume is necessary.

Examples of spike-in volumes for different categories of parent and spike-in panel

Number of primer pairs per pool			Concentration (nM)			Volumes for spiking (µL)		Final concentration (nM)	
Parent	Spike-in	Combined	Parent	Spike-in	Target (combined)	Parent	Spike-in	Parent	Spike-in
50	20	70	400	2,500	400	160	25.6	345	345
80	60	140	100	2,500	100	160 (diluted)	6.4	96.2	96.2
3,000	120	3,120	100	2,500	98.5	160	6.4	96.2	96.2

Prepare DNA target amplification reactions for panels with 3 or 4 primer pools

Use the following protocols to set up target amplification reactions if your Ion AmpliSeq™ DNA panel has 3 or 4 primer pools.

Prepare DNA target amplification reactions—3 primer pools

If you are using a DNA panel with 3 primer pools, set up three 10-µL target amplification reactions, similar to panels with 2 primer pools, then combine after target amplification to yield a total volume of 30 µL.

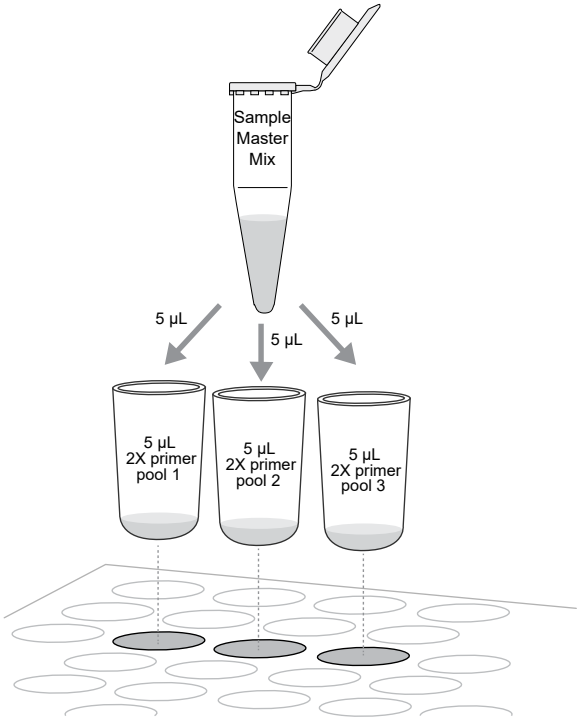
1. For DNA panels with 3 primer pools, use the following table to prepare for each sample a target amplification master mix without primers in a 1.5-mL tube.

   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.

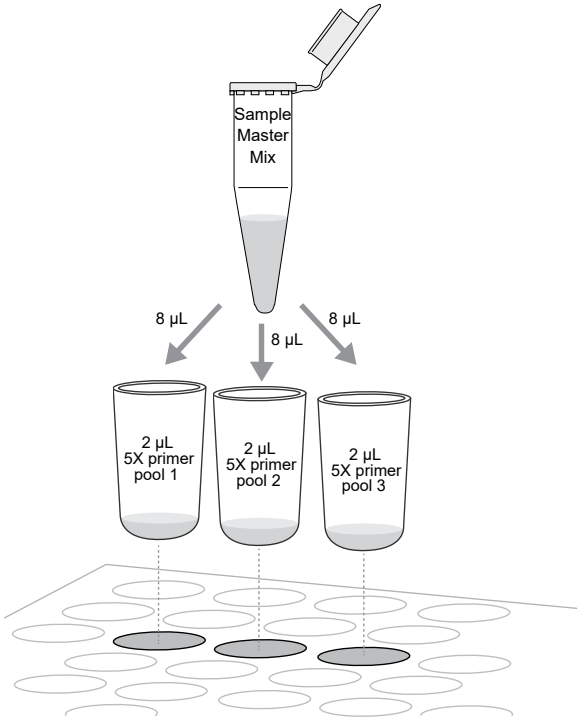
Component	Volume
3-pool panels at 2X concentration	
5X Ion AmpliSeq™ HiFi Mix (red cap)	7 µL
DNA (3–100 ng), or Direct FFPE DNA preparation	≤10.5 µL
Nuclease-free water	to 17.5 µL
3-pool panels at 5X concentration	
5X Ion AmpliSeq™ HiFi Mix (red cap)	7 µL
DNA (3–100 ng), or Direct FFPE DNA preparation	≤21 µL
Nuclease-free water	to 28 µL

2. Mix thoroughly by pipetting up and down 5 times, then transfer sample-specific master mixes to 3 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 3 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 3 primer pools.

3-pool panels at 2X concentration



3-pool panels at 5X concentration



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™ Clear Adhesive Film, then place a MicroAmp™ Optical Film Compression Pad on the plate. Proceed to "Amplify the targets" on page 24.

   See the following instructions for modifications to the protocol after target amplification when you use a 3-pool panel.

   To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

Combine target amplification reactions

For 3 primer pools, combine the target amplification reactions after amplification and before proceeding to the digestion, adapter ligation, and purification steps.

1. After amplification, briefly centrifuge the plate to collect the contents, then carefully remove the plate seal.

2. For each sample, combine the 10-µL target amplification reactions. The total volume for each sample should be 30 µL.

   For 3-pool panels, increase the volumes of FuPa Reagent, ligation components, and MagMAX™ Pure Bind Beads volumes by 50% in the next steps.

Primer pools	FuPa Reagent	Switch Solution	Barcode adapters	DNA Ligase	MagMAX™ Pure Bind Beads
3	3 µL	6 µL	3 µL	3 µL	67.5 µL

Prepare DNA target amplification reactions—4 primer pools

If you are using a DNA panel with 4 primer pools, set up four 10-µL target amplification reactions, similar to panels with 2 primer pools, then combine after target amplification to yield a total volume of 40 µL.

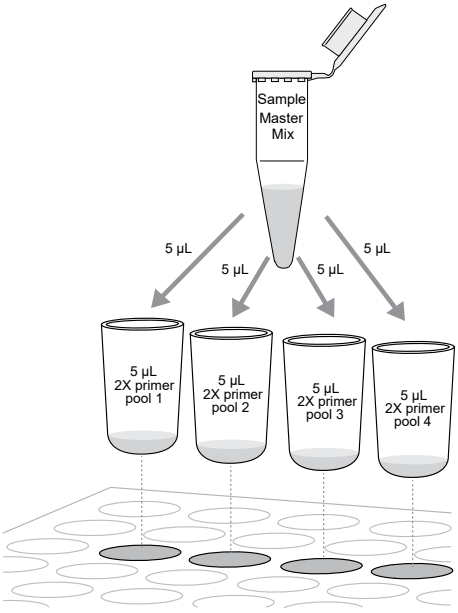
1. For DNA panels with 4 primer pools, use the following table to prepare for each sample a target amplification master mix without primers in a 1.5-mL tube.

   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.

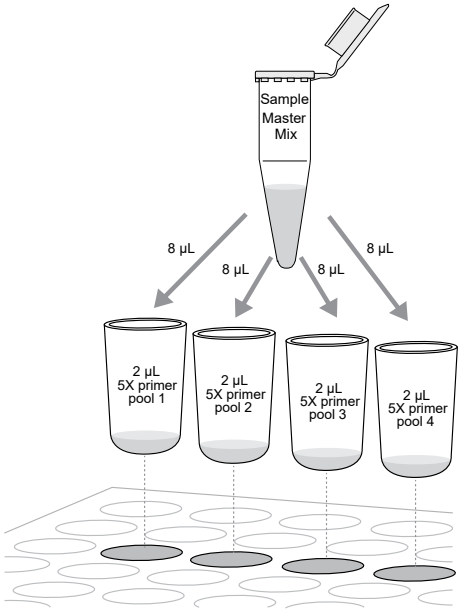
Component	Volume
4-pool panels at 2X concentration	
5X Ion AmpliSeq™ HiFi Mix (red cap)	9 µL
DNA (4–100 ng), or Direct FFPE DNA preparation	≤13.5 µL
Nuclease-free water	to 22.5 µL
4-pool panels at 5X concentration	
5X Ion AmpliSeq™ HiFi Mix (red cap)	9 µL
DNA (4–100 ng), or Direct FFPE DNA preparation	≤27 µL
Nuclease-free water	to 36 µL

2. Mix thoroughly by pipetting up and down 5 times, then transfer sample-specific master mixes to 4 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 4 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 4 primer pools.

4-pool panels at 2X concentration



4-pool panels at 5X concentration



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™ Clear Adhesive Film, then place a MicroAmp™ Optical Film Compression Pad on the plate.

   To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

   Proceed to “Amplify the targets” on page 24.

4. Combine the target amplification reactions.

   See “Combine target amplification reactions” on page 70.

5. Proceed to step 2 of “Partially digest amplicons” on page 25.

Combine target amplification reactions

For 4 primer pools, combine the target amplification reactions after amplification and before proceeding to the digestion, adapter ligation, and purification steps.

1. After amplification, briefly centrifuge the plate to collect the contents, then remove the plate seal.
2. For each sample, combine the 10-µL target amplification reactions. The total volume for each sample should be 40 µL.

   For 4-pool panels, double the volumes of FuPa Reagent, ligation components, and MagMAX™ Pure Bind Beads in the next steps.

Primer pools	FuPa Reagent	Switch Solution	Barcode Adapters	DNA Ligase	MagMAX™ Pure Bind Beads
4	4 µL	8 µL	4 µL	4 µL	90 µL

Reverse transcribe RNA with the SuperScript™ VILO™ cDNA Synthesis Kit

If you are using the SuperScript™ VILO™ cDNA Synthesis Kit to reverse transcribe RNA, use this procedure to set up and run your reactions.

1. Warm the 5X VILO™ Reaction Mix to room temperature for at least 20 minutes, then vortex to mix. If there is any visible precipitate, mix further by vortexing until the 5X VILO™ Reaction Mix is completely resuspended.

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

3. 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**

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

5. Place a MicroAmp™ Optical Film 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 minutes
85°C	5 minutes
10°C	Hold

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

6. Gently tap the plate on the bench to ensure that reactions are at the bottom of the wells, or if possible, briefly centrifuge the plate to collect the contents. Proceed to the next step.

   - If you are using an RNA panel with 1 primer pool, proceed to “Prepare cDNA target amplification reactions—1 primer pool” on page 32
   - If you are using an RNA panel with 2 primer pools, proceed to “Prepare cDNA target amplification reactions—2 primer pools” on page 32

(Optional) Remove 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, heat-labile(UDG) before target amplification.

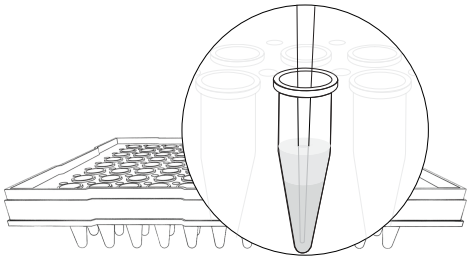
1. Add 1–2 units of UDG to the Direct FFPE DNA lower aqueous phase of each sample after the 15-minute incubation at 65°C.
2. Set a 20-µL pipette to 15 µL, then mix the lower phase by pipetting up and down 10 times.
3. *(Optional)* If necessary, briefly centrifuge to collect the contents, then reseparate the liquid phases in the bottom of the tube.
4. Seal the plate, then incubate at 37°C for 5 minutes, followed by 5 minutes at 65°C.

For the next step, see the *Remove aliquot for library preparation* topic in the *Ion AmpliSeq™ Direct FFPE DNA Kit User Guide* (Pub. No. MAN0014881)

Remove an aliquot for library preparation

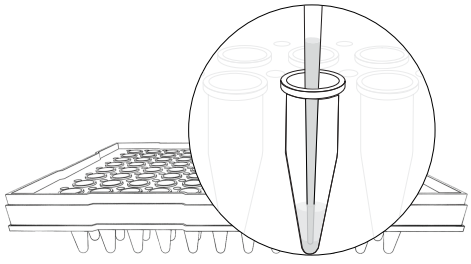
1. Set a 20-µL pipette to 15 µL, depress the plunger to the first stop and insert the pipet tip into the lower (aqueous) phase, then pipet up and down to mix the sample.

   Mixing the sample before removal helps ensure a homogeneous sample before removing aliquots. Avoid disturbing the upper phase and interface while mixing.



2. Based on the number of primer pools and reaction size, remove 6–20 µL 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.

   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.

3. Carefully inspect each transferred sample aliquot for air bubbles. Remove any air bubbles by gently pipetting up and down.

To complete the assembly of the target amplification reactions, proceed to the *Prepare DNA target amplification reactions* protocol that is appropriate to the panel that you are using.

Note: The Direct FFPE DNA preparation can be stored for up to 6 months at –20°C before library preparation.

(Optional) Remove deaminated bases from purified FFPE DNA

IMPORTANT! If using DNA isolated from sources other than FFPE tissue, proceed directly to "Prepare DNA target amplification reactions" on page 20.

FFPE preservation methods can lead to significant cytosine deamination of the isolated DNA, resulting in decreased sequencing quality. Deaminated cytosine (uracil) bases can be enzymatically removed by treatment with Uracil DNA Glycosylase (UDG) before the target amplification reaction.

1. For each FFPE DNA sample, add the following components to a single well of a 96-well PCR plate.

Component	Volume
20 ng FFPE DNA	≤9 µL
UDG, heat-labile	1 µL
Low TE	to 10 µL

2. Mix the reaction by pipetting at least half the total volume up and down at least 5 times, then seal the plate with MicroAmp™ Clear Adhesive Film.

   Alternatively, seal the plate, vortex for 5 seconds to mix the reactions, then briefly centrifuge to collect the contents.

   To prevent evaporation during UDG treatment, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

3. Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate into the thermal cycler, then run the following program.

Temperature	Time
37°C	2 minutes
50°C	10 minutes
4°C	Hold (≤1 hour)

4. Remove the plate from the thermal cycler, then briefly centrifuge to collect the contents.

   STOPPING POINT Reactions can be stored at –20°C long term.

5. Carefully remove the plate seal, then proceed immediately to "Prepare DNA target amplification reactions" on page 20. Add 5 µL of the UDG treated DNA to the target amplification reaction (for 10 ng input).

(Optional) Qubit™ Fluorometer—Quantify the FFPE DNA

When using the Ion AmpliSeq™ Direct FFPE DNA Kit, the DNA concentration can be estimated using a Qubit™ 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 for all samples and standards by diluting Qubit™ dsDNA HS Reagent 1:200 in Qubit™ dsDNA HS Buffer.
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 of each sample and standard on the Qubit™ Fluorometer.
6. *(Qubit™ 2.0 Fluorometer only.)* Calculate the concentration of the undiluted sample by multiplying by the dilution factor. Alternatively, use the **Calculate Stock Conc.** feature on your instrument.

Proceed to the "Prepare DNA target amplification reactions" protocol that is appropriate to the panel that you are using; adjust the volume down to achieve the desired DNA input.

C Strategies for combining Ion AmpliSeq™ libraries

This section describes various strategies for combining Ion AmpliSeq™ libraries with unique barcodes to a standard final concentration of 100 pM for sequencing together on a single chip. See "Ion Chip™ capacities for Ion AmpliSeq™ DNA libraries sequenced at equal depths" on page 79, and "Ion Chip™ capacities for Ion AmpliSeq™ RNA libraries" on page 80 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™ Barcode Adapters 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.

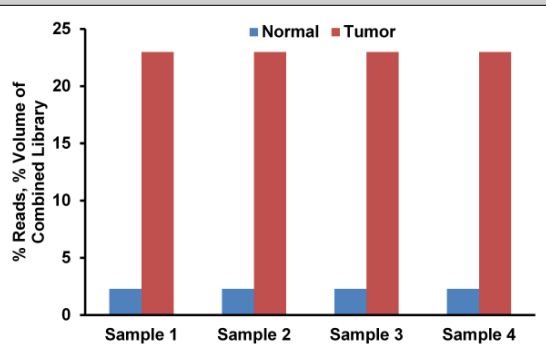
For example, if 16 libraries prepared with the same Ion AmpliSeq™ DNA or RNA panel are combined in a single templating and sequencing reaction, combine the libraries as follows.

1. Dilute all individual libraries to 100-pM concentration.
2. Add 10 µL of each of the 16 libraries to 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. 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 per 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**	

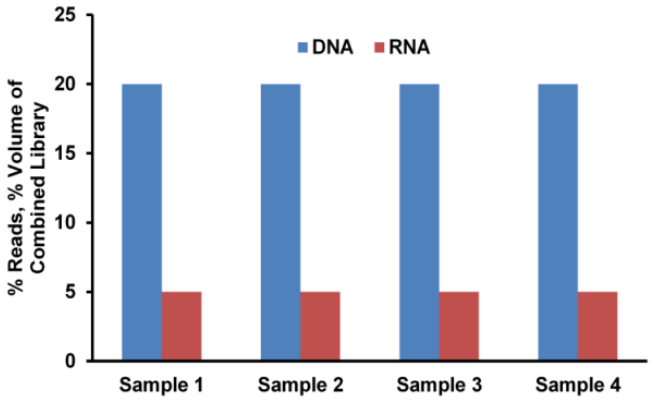
For the example above, if 8 libraries are prepared with the same Ion AmpliSeq™ panel, combine the libraries as follows.

1. Dilute all individual libraries to a 100 pM concentration.
2. Add 23 µL of each “tumor” library to a single tube.
3. Add 2.3 µL of each “normal” library into the same tube.
4. Mix the combined libraries and proceed to templating and sequencing.

Combine DNA and RNA libraries to obtain different numbers of reads

When 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**	

For the example above, if 8 paired DNA/RNA libraries are prepared, combine the libraries as follows.

1. Dilute all individual libraries to a 100 pM concentration.
2. Add 20 µL of each DNA library to a single tube.
3. Add 5 µL of each RNA library into the same tube.
4. Mix the combined libraries, then proceed to templating and sequencing.

Combine libraries prepared using different panels for equal coverage

Use this method to combine libraries from different panels to be sequenced on a single chip at approximately the same depth. To prepare 100 µL of combined library from libraries prepared from two different panels, do the following.

1. Dilute each library to ~100-pM concentration.
2. Combine the libraries using the following formula.

- Volume (µL) of library from Panel 1 = $100\ \mu L \times \frac{\text{Number of primer pairs in Panel 1}}{\text{Total number of primer pairs in Panels 1 and 2}}$

- Volume (µL) of library from Panel 2 = $100\ \mu L \times \frac{\text{Number of primer pairs in Panel 2}}{\text{Total number of primer pairs in Panels 1 and 2}}$

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

The number of combined libraries that can be accommodated in a single sequencing run depends on the chip, the balance of barcoded library concentration, and the coverage that is 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 table serve as a guide for approximate capacities. As the number of libraries per chip increases, it becomes more difficult to balance the reads between libraries. In addition, libraries from FFPE tissue tend to produce more variable results. We suggest combining fewer libraries initially and determining real limits empirically.

	Ion Chip™									
	Ion 510™		Ion 520™		Ion 530™		Ion 540™		Ion 550™	
Average depth	**150X**	**2500X**	**150X**	**2500X**	**150X**	**2500X**	**150X**	**2500X**	**150X**	**2500X**
Expected coverage	**95% >30X**	**95% >500X**	**95% >30X**	**95% >500X**	**95% >30X**	**95% >500X**	**95% >30X**	**95% >500X**	**95% >30X**	**95% >500X**
Amplicons per library	**Approximate number of libraries per chip**									
12–24	272	26	>384	48	>384	152	>384	>384	>384	>384
25–48	152	14	272	26	>384	84	>384	272	>384	>384
49–96	84	8	152	14	>384	48	>384	152	>384	243
97–192	48	4	84	8	272	26	>384	84	>384	134
193–384	26	2	48	4	152	14	>384	48	>384	77
385–768	14	1	26	2	84	8	272	26	>384	41
769–1,536	8	—	14	1	48	4	152	14	243	22
1,537–3,072	4	—	8	—	26	2	84	8	134	13
3,073–6,144	2	—	4	—	14	1	48	4	77	7
6,145–12,288	1	—	2	—	8	—	26	2	41	3
12,289–24,576	—	—	1	—	4	—	14	1	22	2
24,577–49,152	—	—	—	—	2	—	8	—	13	1

Ion Chip™ capacities for Ion AmpliSeq™ RNA libraries

If there is a user guide for your RNA panel, follow the recommendations in the user guide for your panel.

The following guidelines can be used for panels without a user guide. These recommendations serve as suggestions. 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. We suggest using the formula for new panels and determining actual multiplexing limits empirically.

- We recommend that you plan for an average of 5,000 reads per amplicon for an Ion AmpliSeq™ RNA library targeting 1–200 genes. The actual sequencing depth that is required depends on the expression levels of the gene targets in your sample RNA, so scale the sequencing depth to accommodate your sample type and research needs.
- For panels containing fusion detection primer pairs, higher library multiplexing is possible because most targets are not present, and therefore do not create library molecules. For most fusion detection assays, only ~250,000 reads per library are required.
- Use the following formula and chip capacity table to provide initial guidance for multiplexing RNA-derived gene expression libraries on Ion sequencing chips.

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

	Ion Chip™				
	Ion 510™	Ion 520™	Ion 530™	Ion 540™	Ion 550™
Chip capacity in reads (M)	2–3	3–5	15–20	60–80	100–130

Example:

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

Sequencing depth desired = 5,000 reads per amplicon

Number of primer pairs = 100

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

D

Ion AmpliSeq™ panels

Order Ion AmpliSeq™ panels using Ion AmpliSeq™ Designer. For more information, visit **AmpliSeq.com**.

Oncomine™ tumor-specific panels

The Ion Torrent™ Oncomine™ tumor-specific panel designs are small curated panels with verified performance that complement the on-market Oncomine™ assays. They provide an end-to-end solution for research of specific tumors such as bladder, prostate, melanoma, and others from FFPE tissue samples.

The Oncomine™ tumor-specific panels offer the following benefits.

- Small tumor-specific panels with relevant content for research
- End-to-end workflow support that includes bioinformatics and reporting
- Low sample input requirement and robust performance from FFPE tissue samples
- Specialized support for assay verification
- Panel customization

A variety of tumor-specific panels are available in Ion AmpliSeq™ Designer. The performance of each panel was verified on oncological research samples. A Copy Number Variant (CNV) baseline is provided with each panel for data analysis in Ion Reporter™ Software for samples that are sequenced on the Ion 530™ Chip or the Ion 540™ Chip.

For more information, visit **https://www.thermofisher.com/us/en/home/clinical/preclinical-companion-diagnostic-development/oncomine-oncology/oncomine-tumor-specific-panels.html**

Ion AmpliSeq™ On-Demand Panels

Ion AmpliSeq™ On-Demand Panels are designs that are suitable for common disease research areas and are customizable at the gene level. The designs include optimized amplicons with verified performance for germline analysis. You can create designs by selecting from a catalogue of > 5,000 pretested genes or browsing the Ion AmpliSeq™ Designer gene repository by research area of interest.

Each Ion AmpliSeq™ On-Demand Panel consists of 2 DNA primer pools, where the number of primer pairs per pool depends on the genes that are selected. The maximum number of genes that can be selected for design creation is 800. Each Ion AmpliSeq™ On-Demand Panel *order* is limited to 500 genes or 15,000 amplicons. The minimum number of amplicons per design is 24.

You can add genes that are not in the On-Demand catalog to your panel as a spike-in panel. Spike-in panels are high concentration made-to-order panels that are used to supplement on-demand designs with genes that are not available on-demand. Spike-in panels are created as 2-pool designs and are limited to 123 amplicons per pool.

Ion AmpliSeq™ On-Demand Panel designs are available in 8- or 32-reaction packs for library preparations on the Ion Chef™ System and in 24- or 96-reaction packs for manual library preparation. The panels support sequencing genomic targets from as little as 10 ng of DNA input.

Ion AmpliSeq™ fixed panels

Ion AmpliSeq™ fixed panels are fixed-design DNA and RNA panels that are created for germline or somatic analysis. These panels are verified for performance. They are suitable for a wide range of research areas, including inherited diseases, cancer research, human identification, infectious diseases, pharmacogenomics, developmental disorders, inflammtion and immune response research, exome, and more.

Two types of fixed panels are available.

- Ion AmpliSeq™ Community Panels are fixed panels that are designed with input from leading disease researchers and are available to ship withing a few weeks. These panels are verified for performance, and facilitate targeted sequencing for your research in cancer, inherited disease, and microbial and infectious disease.

  When ordered in the tubes-only format, Ion AmpliSeq™ Community Panels contain 750 reactions (1 pool) or 1,500 reactions (2 pool) for panels with up to 96 amplicons; or 3,000 reactions (1 pool) or 6,000 reactions (2 pool) for panels with more than 96 amplicons.

  When ordered in the tubes plus 384-well plate format, Ion AmpliSeq™ Community Panels contain 2,250 reactions (1 pool) or 4,500 reactions [2 pool] for panels with up to 96 amplicons; or 9,000 reactions (1 pool) or 18,000 reactions [2 pool] for panels with more than 96 amplicons. In this format reactions are split between tubes (1/3 rd) and plates (2/3 rd).
- Ion AmpliSeq™ Ready-to-Use Panels are fixed panels that are designed by Thermo Fisher Scientific researchers and are in stock and ready to ship immediately.

  The number of reactions of Ion AmpliSeq™ Ready-to-Use Panels depends on the panel. You can view the number of available reactions on each product page on **www.thermofisher.com**.

Ion AmpliSeq™ Made-to-Order Panels

Ion AmpliSeq™ Made-to-Order Panels are designs that are customizable for any genome at the amplicon level. Panels can be designed against a number of preloaded reference genomes, or for any other organism by uploading a FASTA file reference sequence.

Each Ion AmpliSeq™ Made-to-Order 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 amplicon.

You can use the plates with panels in the following ways.

- Remake the entire panel.
- Remake a smaller panel containing a subset of primer pairs.
- Combine two panels.
- Make a 50X spike-in panel to add to other panels.

When ordered in the tubes-only format, Ion AmpliSeq™ Made-to-Order Panels contain 750 reactions (1 pool) or 1,500 reactions (2 pool) for panels with up to 96 amplicons; or 3,000 reactions (1 pool) or 6,000 reactions (2 pool) for panels with more than 96 amplicons.

When ordered in the tubes plus 384-well plate format, Ion AmpliSeq™ Made-to-Order Panels contain 2,250 reactions (1 pool) or 4,500 reactions [2 pool] for panels with up to 96 amplicons; or 9,000 reactions (1 pool) or 18,000 reactions [2 pool] for panels with more than 96 amplicons. In this format reactions are split between tubes (1/3 rd) and plates (2/3 rd).

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 (per primer).

- For panels with up to 96 primer pairs per pool, combine and dilute to 400 nM per primer pair (2X:400 nM).

  To prepare at 5X, combine and dilute to 1,000 nM per primer pair (5X:1,000 nM).
- For panels with 97–3,072 primer pairs per pool, combine and dilute to 100 nM per primer pair (2X:100 nM).

  For panels up to 1,228 primer pair, to prepare at 5X, combine and dilute to 250 nM per primer pair (5X:250 nM).

For example, use these steps to create a primer pool with 2 µL of each primer pair.

1. Vortex the sealed primer plate, then briefly centrifuge to collect the contents.
2. Remove the plate seal, then 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
2X, 400 nM (12–96 primer pairs)	Add Low TE to a final volume of 1.536 mL. Can be prepared at 5X, 1,000 nM by increasing the final volume to 0.614 mL.
2X, 100 nM (97–3,072 primer pairs)	Add Low TE to a final volume of 6.140 mL. For panels up to 1,228 primer pairs, can be prepared at 5X, 250 nM by increasing the final volume to 2.456 mL.
50X spike-in panel, 2,500 nM (1–123 primer pairs)	Add Low TE to a final volume of 0.246 mL.

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

Ion AmpliSeq™ Sample ID Panel

Use the Ion AmpliSeq™ Sample ID Panel to add (spike in) content to Ion AmpliSeq™ Ready-to-Use Panel, Ion AmpliSeq™ Made-to-Order Panel, or Ion AmpliSeq™ On-Demand Panel. For example, use the Ion AmpliSeq™ Sample ID Panel to add amplicons that create a unique identifier for human gDNA samples.

Panel	Conc.	Approx. library size (with adapters)	Quantity	No. of primer pairs	Storage[1]
Ion AmpliSeq™ Sample ID Panel (Cat. No. 4479790)	20X	150–220 bp	1 tube (96 rxns)	9	–30°C to –10°C

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

Use the Ion AmpliSeq™ Sample ID Panel

If you use an Ion AmpliSeq™ On-Demand Panel, Ion AmpliSeq™ Ready-to-Use Panel, Ion AmpliSeq™ Made-to-Order Panel, or Ion AmpliSeq™ Community Panel in a manual Ion AmpliSeq™ protocol, use the following procedure to create a sample signature in your panel.

If you use the Ion AmpliSeq™ Kit for Chef DL8, use the following procedure to create a sample signature in your panel.

1. Add 1 µL of the Ion AmpliSeq™ Sample ID Panel to the target amplification reaction.
   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.

2. When creating a new Planned Run in the Torrent Browser Planned Run Wizard, select the following settings.
   a. In the **Kits** screen, select the **Control Sequence**, then select **Ion AmpliSeq Sample ID panel** from the list.

b. In the **Plugins** screen, select the **coverageAnalysis** plugin checkbox, then click **Configure**.

c. In the configuration dialog box, select the **Sample Tracking** checkbox.

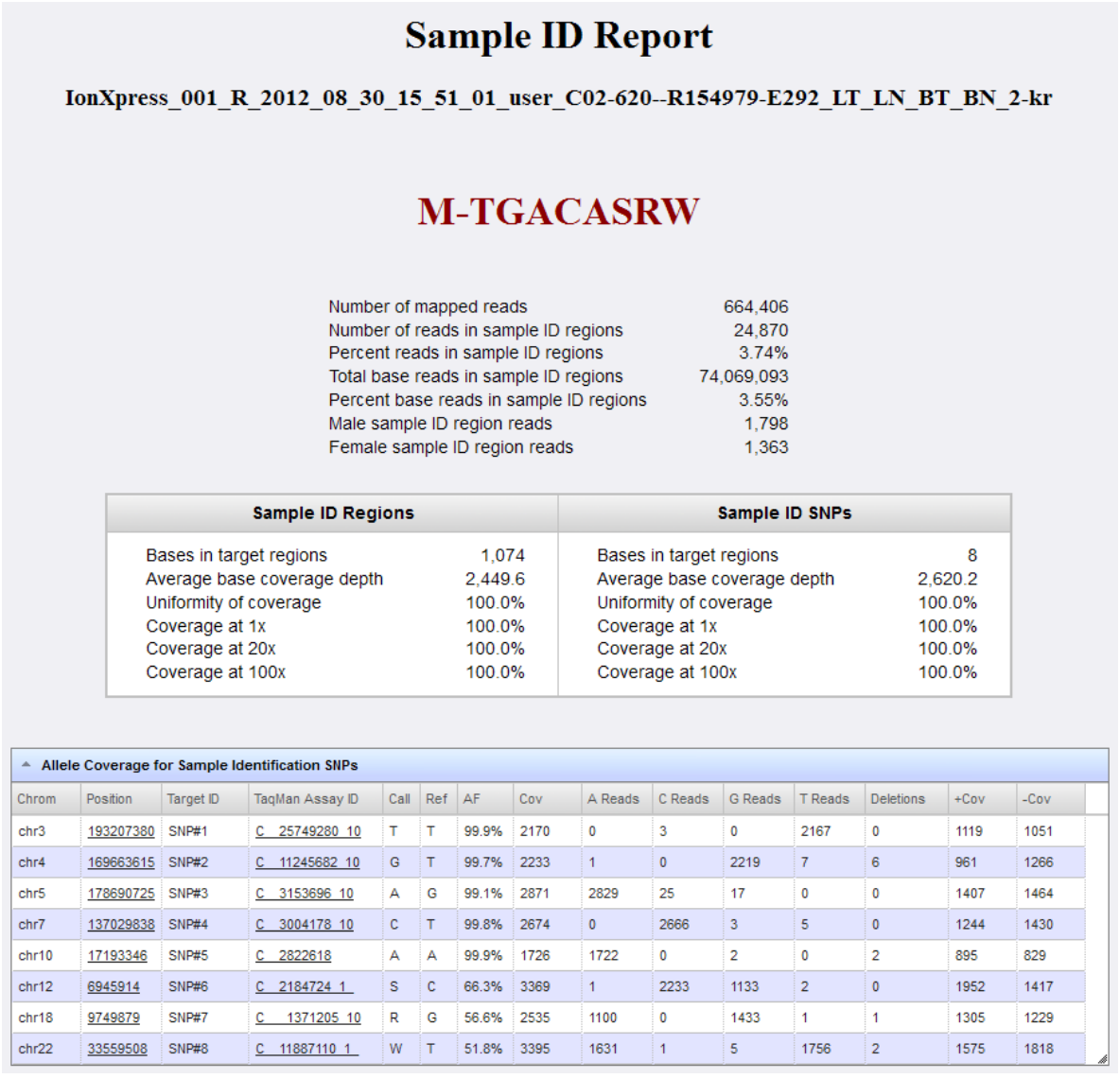
When **Sample Tracking** is selected, the analysis produces a statistic for reads that are mapped to Sample ID targets so that the level of off-target reads is accurately represented in the **Coverage Analysis Report**.

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

d. In the **Plugins** screen, select the **sampleID plugin**.

A **Sample ID Report** is generated after the run.

3. After 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

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, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Chemical safety

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

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer 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 needed) 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! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

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

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  cdc.gov/labs/bmbl
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  who.int/publications/i/item/9789240011311

Documentation and support

Related documentation

Document	Publication number
Ion AmpliSeq™ Library Kit Plus Quick Reference—Library Preparation Using the Ion Library Equalizer™ Kit	MAN0017004
Ion AmpliSeq™ Library Kit Plus Quick Reference—qPCR Quantification	MAN0017005
Ion AmpliSeq™ Library Kit Plus Quick Reference—Qubit™ Fluorometer and Agilent™ 2100 Bioanalyzer™ Instrument Quantification	MAN0017006
Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide	MAN0013432
Bisulfite Methylation Library Production using the Ion AmpliSeq™ Library Kit Plus User Bulletin	MAN0017662
Bisulfite Methylation Library Production using the Ion AmpliSeq™ Kit for Chef DL8 User Bulletin	MAN0017892
Prepare Ion AmpliSeq™ Libraries Using the Tecan™ Freedom EVO™ NGS Workstation User Bulletin	MAN0010822
Ion AmpliSeq™ Transcriptome Human Gene Expression Kit User Guide	MAN0010742
Ion AmpliSeq™ Transcriptome Mouse Gene Expression Kit User Guide	MAN0017343
Ion AmpliSeq™ Exome RDY Library Preparation User Guide	MAN0010084
Demonstrated Protocol: Sample Quantification for Ion AmpliSeq™ Library Preparation Using the TaqMan™ RNase P Detection Reagents Kit	MAN0007732
Ion AmpliSeq™ Direct FFPE DNA Kit User Guide	MAN0014881
Qubit™ dsDNA HS Assay Kits User Guide	MAN0002326
Ion Reporter™ Software 5.20 User Guide	MAN0028321
Torrent Suite™ Software 5.18 User Guide	MAN0026163
Ion S5™ Sequencer User Guide	MAN0017528
Ion 550™ Kit – Chef User Guide	MAN0017275
Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide	MAN0016854
Ion 540™ Kit – Chef User Guide	MAN0010851

(continued)

Document	Publication number
Ion AmpliSeq™ & Ion AmpliSeq™ HD Custom Assay User Guide	MAN0028005
Ion AmpliSeq™ Designer Help	available at **AmpliSeq.com**

For additional documentation, see “Customer and technical support”.

Customer and technical support

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- Product documentation
  - User guides, manuals, and protocols
  - 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

Life Technologies Corporation and its affiliates warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have questions, contact Life Technologies at **www.thermofisher.com/support**.