Ion AmpliSeq™ Library Kit 2.0

USER GUIDE

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

Catalog Numbers 4475345, 4480441, 4480442
Publication Number MAN0006735
Revision F.0

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

ThermoFisher SCIENTIFIC

Life Technologies Corporation | 5781 Van Allen Way | Carlsbad, CA 92008
For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

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

DISCLAIMER: TO THE EXTENT ALLOWED BY LAW, THERMO FISHER SCIENTIFIC INC. AND/OR ITS AFFILIATE(S) WILL NOT BE LIABLE FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE, OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING YOUR USE OF IT.

Revision history: Pub. No. MAN0006735

Revision	Date	Description
F.0	30 January 2019	• Support added for Ion 510™ and Ion 550™ Chips • Support added for Ion GeneStudio™ S5 Systems • Support added for Ion Torrent™ Dual Barcode Kit 1–96 • Hold and Denature temperature recommended for amplification of targets for RNA panels changed from 99°C to 98°C • Added instructions for removing deaminated bases from purified FFPE DNA. • Instructions clarified for Ion AmpliSeq™ Made-to-Order Panels, spike-in panels, and Ion AmpliSeq™ Sample ID Panel • Style updates
E.0	24 May 2017	• Title of user guide modified • Custom Panels changed to "Made-to-Order Panels" • Storage recommendation of Direct FFPE DNA preparation changed from 3 months to 6 months at –20°C • Lower limit of gDNA added to target amplification master mixes for multi-primer pool panels changed from 1 ng total to 1 ng per primer pool • Last two rows of the amplification cycle table merged, anneal/extension time column added to table, and anneal/extension time recommendations modified for higher plexy panels (see "Amplify the targets" on page 27 and "Amplify the targets" on page 36) • Hold time at 10°C after ligation reaction clarified • Instructions added to seal plate with adhesive film optionally after addition of Equalizer™ Beads (see "Add Equalizer™ Beads and wash" on page 44) • Tip added for amplifying libraries in the presence of Agencourt™ AMPure™ XP beads (see "Tips" on page 54)
D.0	14 March 2017	• Chapter structure reorganized for ease of use • Reverse transcription reaction conditions updated for use of the SuperScript™ IV VILO™ Master Mix • Guidance added for target amplification conditions for 375 bp amplicon designs • Ion Chip capacity table for Ion AmpliSeq™ DNA libraries updated

Important Licensing Information: These products may be covered by one or more Limited Use Label Licenses. By use of these products, you accept the terms and conditions of all applicable Limited Use Label Licenses.

Trademarks: All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified.

Bioanalyzer and Agilent are trademarks of Agilent Technologies, Inc. TaqMan is a registered trademark of Roche Molecular Systems, Inc., used under permission and license. Eppendorf LoBind is a trademark of Eppendorf AG. Freedom EVO is a trademark of Tecan Group Ltd. Agencourt and AMPure are trademarks of Beckman Coulter, Inc.

©2019 Thermo Fisher Scientific Inc. All rights reserved.

Contents

CHAPTER 1 Product information 7

Product description 7
- Ion AmpliSeq™ Kit for Chef DL8 7
- Automation protocols 7

Ion AmpliSeq™ Direct FFPE DNA Kit 8

Ion AmpliSeq™ Library Kits 8
- Ion AmpliSeq™ Library Kit 2.0 8
- Ion AmpliSeq™ Library Kit 2.0-96LV or -384LV 9

Ion Torrent™ Dual Barcode Kit 1–96 9

IonCode™ Barcode Adapters 1–384 Kit 10

Ion Xpress™ Barcode Adapters Kits 10

Ion Library Equalizer™ Kit 11

Ion AmpliSeq™ Panels 12
- Ion AmpliSeq™ Ready-to-Use Panels 12
- Ion AmpliSeq™ Made-to-Order and Community Panels 12
- Ion AmpliSeq™ On-Demand Panels 13
- Ion AmpliSeq™ Spike-in panels 13

Oncomine™ panels 13

Ion AmpliSeq™ panel kits 14

Required materials not supplied 15

Recommended materials and equipment 16

Ion AmpliSeq™ workflow starting from genomic DNA or RNA 18

Procedure overview 19

CHAPTER 2 Prepare Ion AmpliSeq™ DNA libraries 20

Procedural guidelines 20

Before each use of the kit 20

Isolate genomic DNA 21
- Guidelines for DNA isolation and quantification 21
- Guidelines for the amount of DNA needed per target amplification reaction 21
- Prepare DNA with the Ion AmpliSeq™ Direct FFPE DNA Kit 22

Set up DNA target amplification reactions 24
- Prepare DNA target amplification reactions—single primer pool 24
- Prepare DNA target amplification reactions — 2 primer pools 25

Amplify the targets 27
Combine target amplification reactions (Only for DNA libraries with 2 primer pools) 28
Partially digest amplicons 28
Ligate adapters to the amplicons and purify 29
Ion Xpress™ adapters only: Combine and dilute adapters 29
Perform the ligation reaction 30
Purify the library 31
Proceed to library equalization or quantification 31

CHAPTER 3 Prepare Ion AmpliSeq™ RNA libraries 32

Procedural guidelines 32
Before each use of the kit 32
Guidelines for RNA isolation, quantification, and input 33
Reverse transcribe RNA 33
Prepare cDNA target amplification reactions—single primer pool 34
Prepare cDNA target amplification reactions—2 primer pools 35
Amplify the targets 36
Combine target amplification reactions (Only for RNA libraries with 2 primer pools) 37
Partially digest amplicons 37
Ligate adapters to the amplicons and purify 38
Ion Xpress™ adapters only: Combine and dilute adapters 38
Perform the ligation reaction 38
Purify the library 39
Proceed to library equalization or quantification 40

CHAPTER 4 Equalize the library 41

Before you begin 41
Amplify the library 42
Wash the Equalizer™ Beads *(if not previously performed)* 43
Add Equalizer™ Capture to the amplified library 43
(Optional) Combine captured libraries 43
Add Equalizer™ Beads and wash 44
Elute the Equalized library 45
(Optional) Combine Equalized libraries 45
Store libraries 45

CHAPTER 5 Quantify the library by qPCR 46

Elute the library 46
Quantify library by qPCR and calculate the dilution factor 46
(Optional) Combine amplicon libraries 48
Store libraries 48

CHAPTER 6 Quantify the amplified library with a Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ instrument 49

Amplify the library 49
Purify the amplified library 50
First-round purification 50
Second-round purification 51
Qubit™ Fluorometer: Quantify the library and calculate the dilution factor 52
Agilent™ 2100 Bioanalyzer™ instrument: Quantify the library and calculate the dilution factor 53
(Optional) Combine amplicon libraries 53
Store libraries 53

APPENDIX A Tips and troubleshooting 54

Tips 54
Modifications to the standard workflow 55
Shortcuts 55
Limited samples 56
Troubleshooting 57
Ion AmpliSeq™ Direct FFPE DNA Kit 57
Library yield and quantification 58
Low amplicon uniformity (DNA only) 60
Other 63

APPENDIX B Supplemental procedures 65

Prepare DNA target amplification reactions for panels with 3 or 4 primer pools 65
Prepare DNA target amplification reactions — 3 primer pools 65
Prepare DNA target amplification reactions — 4 primer pools 67
Reverse transcribe RNA with the SuperScript™ VILO™ cDNA Synthesis Kit 70
(Optional) Remove deaminated bases from Direct FFPE DNA 71
(Optional) Remove deaminated bases from purified FFPE DNA 71
(Optional) Qubit™ Fluorometer: Quantify the FFPE DNA 72

APPENDIX C Strategies for combining Ion AmpliSeq™ libraries .. 73

Combine libraries prepared with one panel for equal depth of coverage 73
Combine libraries prepared from one panel to vary depth of coverage 74
Combine DNA and RNA libraries to obtain different numbers of reads 75
Combine libraries prepared using different panels for equal coverage 76
Ion Chip capacities for Ion AmpliSeq™ DNA libraries sequenced at equal depths 77
Ion Chip capacities for Ion AmpliSeq™ RNA libraries 78

APPENDIX D Ion AmpliSeq™ Made-to-Order Panels 79

Prepare primer pools from plates 79

APPENDIX E Expand any Ion AmpliSeq™ panel by adding a spike-in panel 81

APPENDIX F Ion AmpliSeq™ Sample ID Panel 82

Using the Ion AmpliSeq™ Sample ID Panel 82

APPENDIX G Data analysis 84

coverageAnalysis plugin 84
coverageAnalysis plugin configuration 84
Review coverageAnalysis plugin results 86
ampliSeqRNA plugin 94
ampliSeqRNA plugin configuration 95
Review ampliSeqRNA plugin results 96

APPENDIX H Safety 98

Chemical safety 99
Biological hazard safety 100

Documentation and support 101

Customer and technical support 101
Limited product warranty 101

1 Product information

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

Product description

This guide covers the following products:

- Ion AmpliSeq™ Direct FFPE DNA Kit (Cat. Nos. A31133, A31136)
- Ion AmpliSeq™ Library Kits:
  - Ion AmpliSeq™ Library Kit 2.0 (Cat. No. 4475345)
  - Ion AmpliSeq™ Library Kit 2.0-96LV (Cat. No. 4480441)
  - Ion AmpliSeq™ Library Kit 2.0-384LV (Cat. No. 4480442)
- Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360)
- IonCode™ Barcode Adapters (Cat. No. A29751)
- Ion Xpress™ Barcode Adapters (various Cat. Nos.)
- Ion Library Equalizer™ Kit (Cat. No. 4482298)
- Ion AmpliSeq™ Ready-to-Use Panels (various Cat. Nos.)
- Ion AmpliSeq™ Made-to-Order Panels (formerly called Custom Panels), and Community Panels (ordered at **AmpliSeq.com**)
- Ion AmpliSeq™ On-Demand Panels (ordered at **AmpliSeq.com**)
- Ion AmpliSeq™ Sample ID Panel (Cat. No. 4479790)

The Ion AmpliSeq™ Library Kits 2.0 include reagents to prepare targeted libraries from DNA or RNA for sequencing on the Ion GeneStudio™ S5, Ion S5™, Ion S5™ XL, Ion PGM™, Ion Proton™ instruments. The kits are designed for use with Ion AmpliSeq™ Ready-to-Use or Made-to-Order Panels and incorporate barcoded adapters so that multiple libraries can be combined and loaded onto a single Ion Chip to minimize the per-sample sequencing cost.

Ion AmpliSeq™ Kit for Chef DL8

The Ion AmpliSeq™ Kit for Chef DL8 provides the reagents and supplies ready to load into an Ion Chef™ instrument for automated preparation of up to 32 Ion AmpliSeq™ libraries (8 libraries per run) with user-supplied primer panels. See the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432) for further details.

Automation protocols

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**.

Ion AmpliSeq™ Direct FFPE DNA Kit

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

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

Ion AmpliSeq™ Library Kits

Ion AmpliSeq™ Library Kit 2.0 contains reagents for the rapid preparation of libraries with 12–24,576 primer pairs per pool from 300–30,000 copies of DNA (1–100 ng of mammalian DNA) or cDNA. These library kits use a plate-based protocol for easy sample handling, and for compatibility with automation in high throughput laboratories.

Ion AmpliSeq™ Library Kit 2.0

The Ion AmpliSeq™ Library Kit 2.0 (Cat. No. 4475345) provides reagents for preparing 8 libraries for 1- or 2-pool panels (5 libraries for 3-pool panels, and 4 libraries for 4-pool panels).

Contents	Amount	Storage
5X Ion AmpliSeq™ HiFi Mix (red cap)	40 µL	–30°C to –10°C
FuPa Reagent (brown cap)	16 µL	
Switch Solution (yellow cap)	32 µL	
DNA Ligase (blue cap)	16 µL	
Ion AmpliSeq™ Adapters (green cap)	16 µL	
Platinum™ PCR SuperMix High Fidelity (black cap)	400 µL	
Library Amplification Primer Mix (white cap)	16 µL	
Low TE	6 mL	15°C to 30°C[1]

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

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

The Ion AmpliSeq™ Library Kit 2.0-96LV (Cat. No. 4480441) provides reagents for preparing 96 libraries for 1- or 2-pool panels (64 libraries for 3-pool panels, and 48 libraries for 4-pool panels). The Ion AmpliSeq™ Library Kit 2.0-384LV (Cat. No. 4480442) provides reagents for preparing 384 libraries for 1- or 2-pool panels (256 libraries for 3-pool panels, and 192 libraries for 4-pool panels).

Contents	Amount		Storage
	96LV	384LV	
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	
Ion AmpliSeq™ Adapters (green cap)	192 µL	4 × 192 µL	
Platinum™ PCR SuperMix High Fidelity (black cap)	3 × 1.6 mL	12 × 1.6 mL	
Library Amplification Primer Mix (white cap)	192 µL	4 × 192 µL	
Low TE	2 × 6 mL	8 × 6 mL	15°C to 30°C[1]

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

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 (20 µL/well)	960 (10 reactions 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 pre-mixed 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	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)	–30ºC to –10ºC

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

The following Ion Xpress™ Barcode Adapters Kits are available:

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

Ion Library Equalizer™ Kit

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

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

Component	Amount	Storage
Equalizer™ Primers (pink cap)	200 µL	2ºC to 8ºC
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™ Ready-to-Use, Made-to-Order, Community, and On-Demand Panels provide pools of primers for amplification of target regions. The primers contain proprietary modifications that enable removal of primer sequences during library preparation for efficient target assessment during sequencing. Multiple primer pools can be used to create overlapping amplicons that enable complete coverage of large targets. Nucleic acid from various sources—including formalin-fixed, paraffin-embedded (FFPE) tissue, and cell-free DNA (cfDNA)—can be used as the starting material.

- Ion AmpliSeq™ Ready-to-Use Panels are available at **thermofisher.com**.
- Ion AmpliSeq™ Ready-to-Use Panels and Community Panels are available at **AmpliSeq.com**. Ion AmpliSeq™ Made-to-Order Panels and On-Demand Panels can be designed and ordered using Ion AmpliSeq™ Designer at **AmpliSeq.com**.

See Appendix D, "Ion AmpliSeq™ Made-to-Order Panels" for further information.

Ion AmpliSeq™ Ready-to-Use Panels

See a complete listing of Ion AmpliSeq™ Ready-to-Use Panels at **AmpliSeq.com**.

Panel	Conc.	Nominal amplicon size	Approx. library size range (with adapters)	Quantity	No. of primer pairs	Storage[1]
gDNA panels (human)						
Ion AmpliSeq™ Cancer Hotspot Panel v2 (Cat. No. 4475346)	5X	175 bp	150–220 bp	1 tube (8 rxns)	207	–30°C to –10°C
Ion AmpliSeq™ Comprehensive Cancer Panel[2] (Cat. No. 4477685)	2X	175 bp	150–250 bp	4 tubes (8 rxns)	~4,000/tube (~16,000 total)	

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

[2] A quantity of 16 reactions can be obtained when using this panel as a 2-primer pool panel. See "Shortcuts" on page 55.

Ion AmpliSeq™ Made-to-Order and Community Panels

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

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

Ion AmpliSeq™ Made-to-Order and Community DNA Panels

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

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

Ion AmpliSeq™ Made-to-Order and Community RNA Panels

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

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

Ion AmpliSeq™ On-Demand Panels

Ion AmpliSeq™ On-Demand Panels are optimized for use with high molecular weight DNA and can be designed and ordered at AmpliSeq.com (**AmpliSeq.com**), by selecting genes using a content selection engine or uploading your own gene list.

If you are preparing libraries using Ion AmpliSeq™ On-Demand Panels, we recommend that you use the Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990), which provides higher performance for these panels. For further information on Ion AmpliSeq™ On-Demand Panels, see the *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003).

Ion AmpliSeq™ Spike-in panels

Spike-in panels enable addition of content to Ion AmpliSeq™ Ready-to-Use, Made-to-Order, or On-Demand Panels. For example, the Ion AmpliSeq™ Sample ID Panel is used 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.

Oncomine™ panels

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

Ion AmpliSeq™ panel kits

See the product page on **www.thermofisher.com** for instructions for any of the following kits.

Panel Kit	Function	Cat. No
Ion AmpliSeq™ Transcriptome Human Gene Expression Kit	Provides gene-level expression information from a single multiplexed panel targeting over 20,000 human genes (> 90% of the RefSeq gene database).	A26325 A26326 A26327
Ion AmpliSeq™ Transcriptome Mouse Gene Expression Kit	Provides gene-level expression information from a single multiplexed panel targeting over 23,000 mouse genes (> 90% of the RefSeq gene database).	A36553 A36554 A36555
Ion AmpliSeq™ Exome RDY Kits	Contain reagents for the rapid preparation of eight exome libraries for sequencing.	A38262 A38264

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**.

Item	Source
One of the following, or equivalent: • SimpliAmp™ Thermal Cycler • Applied Biosystems™ 2720 Thermal Cycler • Veriti™ 96-Well Thermal Cycler • ProFlex™ 96-well PCR System • GeneAmp™ PCR System 9700[1] or Dual 96-well Thermal Cycler	Various
One of the following: • Ion Library Equalizer™ Kit • Ion Library TaqMan® Quantitation Kit and real-time PCR instrument • Qubit™ 4 Fluorometer[2] and the Qubit™ dsDNA HS Assay Kit (DNA), or Qubit™ RNA HS Assay Kit (RNA) • Agilent™ 2100 Bioanalyzer™ and Agilent™ High Sensitivity DNA Kit	4482298 4468802 Q33226 Agilent G2939AA, 5067-4626
One of the following: • IonCode™ Barcode Adapters 1–384 Kit • Ion Xpress™ Barcode Adapters Kit • 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
Eppendorf™ DNA LoBind™ Microcentrifuge Tubes, 1.5-mL	13-698-791 (**fisherscientific.com**)
Agencourt™ AMPure™ XP Kit	NC9959336, NC9933872 (**fisherscientific.com**)
DynaMag™–96 Side Magnet, or other plate magnet	12331D
Nuclease-free Water	AM9932
Ethanol, Absolute, Molecular Biology Grade	BP2818500 (**fisherscientific.com**)

Item	Source
Pipettors, 2–200 µL, and low-retention filtered pipette tips	(fisherscientific.com)
(RNA only) SuperScript™ IV VILO™ Master Mix *or* SuperScript™ VILO™ cDNA Synthesis Kit	 11756050 11754050

[1] Supported but no longer available for purchase.
[2] Qubit™ 2.0 Fluorometer and later are supported.

Recommended materials and equipment

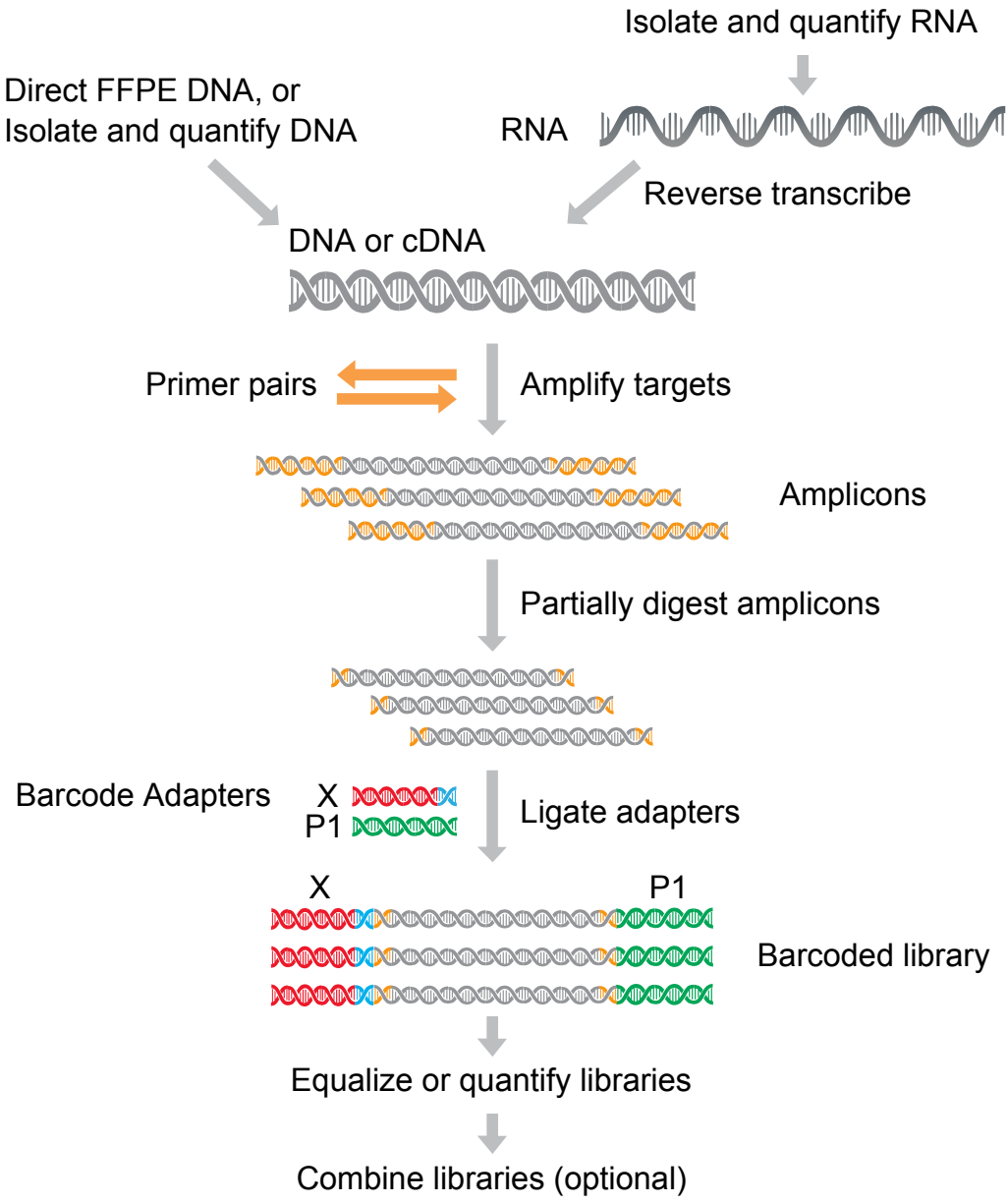
Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Additional equipment	
Real-time PCR instrument (e.g., Applied Biosystems™ 7900HT, 7500, StepOne™, StepOnePlus™, ViiA™ 7, QuantStudio™ 3, QuantStudio™ 5, QuantStudio™7 , or QuantStudio™ 12K Flex Real–Time PCR Systems)	Various
Fisher Scientific™ Mini Plate Spinner Centrifuge, or equivalent 96-well plate centrifuge	14-100-143 (fisherscientific.com)
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	K182000
Nucleic acid quantification	
TaqMan® RNase P Detection Reagents Kit *(Recommended for DNA only)*	4316831
Qubit™ 4 Fluorometer[1] and the Qubit™ dsDNA HS Assay Kit (DNA), or Qubit™ RNA HS Assay Kit (RNA)	Q33226
Additional material for the Ion AmpliSeq™ Direct FFPE DNA Kit	
(Optional) Uracil DNA Glycosylase (UDG)	18054015, EN0361

Item	Source
Additional material for purified FFPE DNA	
Uracil DNA Glycosylase (UDG), heat labile	78310100UN
Controls	
AcroMetrix™ MultiMixC FFPE Control	957186
AcroMetrix™ KRAS FFPE Process Controls	950450
AcroMetrix™ Oncology Hotspot Control	969056
Control DNA (CEPH 1347-02)	403062

[1] Qubit™ 2.0 Fluorometer and later are supported.

Ion AmpliSeq™ workflow starting from genomic DNA or RNA



Procedure overview

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

   Note: The Ion Library Equalizer™ Kit offers the greatest convenience, but can result in a higher proportion of libraries with low yield and read number when sample quality or quantity is low. Quantitative PCR is the most simple and robust workflow, and is recommended for libraries from RNA and samples of unknown quality or quantity. Qubit™ fluorometry is the most economical, but lacks specificity. Agilent™ 2100 Bioanalyzer™ assessment generates the most information for troubleshooting.
7. When barcode adapters are used, libraries can be combined in various ways before sequencing. Combining libraries maximizes chip use while minimizing cost and labor. See Appendix C, “Strategies for combining Ion AmpliSeq™ libraries“ for more information.

2 Prepare Ion AmpliSeq™ DNA libraries

- Procedural guidelines 20
- Before each use of the kit 20
- Isolate genomic DNA 21
- Set up DNA target amplification reactions 24
- Amplify the targets 27
- Combine target amplification reactions (Only for DNA libraries with 2 primer pools) 28
- Partially digest amplicons 28
- Ligate adapters to the amplicons and purify 29

Procedural guidelines

- Minimize freeze-thaw cycles of Ion AmpliSeq™ panels by aliquoting as needed for your experiments. Panels can be stored at 4°C for one year.
- Use good laboratory practices to minimize cross-contamination of products. If possible, perform PCR setup in an area or room that is free of amplicon contamination. Always change pipette tips between samples.
- Use a calibrated thermal cycler specified in "Required materials not supplied" on page 15.
- 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.

Before each use of the kit

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

Isolate genomic DNA

Guidelines for DNA isolation and quantification

- See "Recommended materials and equipment" on page 16 for recommended kits for isolating gDNA.
- We recommend the TaqMan® RNase P Detection Reagents Kit (Cat. No. 4316831) for quantifying amplifiable human genomic DNA (see *Demonstrated Protocol: Sample Quantification for Ion AmpliSeq™ Library Preparation Using the TaqMan® RNAse P Detection Reagents Kit* (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 "Prepare DNA with the Ion AmpliSeq™ Direct FFPE DNA Kit" on page 22 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 using 1 ng gDNA (300 copies) only with high-quality, well-quantified samples.
- Ion AmpliSeq™ On-Demand Panels and some Ion AmpliSeq™ Ready-to-Use, Made-to-Order, and 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 single- and 2-primer pool panels.
- The maximum volume of DNA per reaction depends on the concentration of the Ion AmpliSeq™ primer pool you are using, the number of primer pools in the panel, and whether you are using a spike-in panel such as the Ion AmpliSeq™ Sample ID Panel. See the "Prepare DNA target amplification reaction" topics on pages 23–25 and in Appendix B for the maximum volume of DNA in target amplification reactions.
- For Ion AmpliSeq™ Direct FFPE DNA samples, library yield can be lower for a small tissue area or for degraded samples. Also, inhibitors such as high melanin content can reduce the efficiency of target amplification.

Prepare DNA with the Ion AmpliSeq™ Direct FFPE DNA Kit

The Ion AmpliSeq™ Direct FFPE DNA Kit (Cat. Nos. A31133 and A31136) bypasses nucleic acid isolation when preparing libraries from FFPE sections on slides. Use the following protocol with the Ion AmpliSeq™ Direct FFPE DNA Kit to prepare DNA from FFPE tissue sections suitable for use in Ion AmpliSeq™ library preparation.

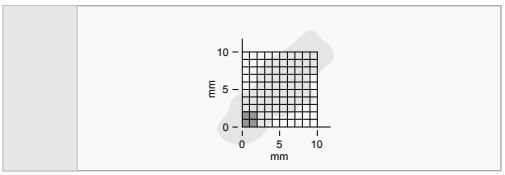
If using purified FFPE DNA, we recommend removing deaminated cytosine (uracil) bases enzymatically by treatment with Uracil DNA Glycosylase (UDG) before the target amplification reaction. See "(Optional) Remove deaminated bases from purified FFPE DNA" on page 71.

Prepare reagents

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

Prepare FFPE DNA

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



Standard microscope slide, 25 × 75 mm.

1. For each sample, pipet 30 µL of Transfer Solution into a single well of a 96-well PCR plate.
2. Using a single 20-µL pipette tip for each sample:
   a. Pipet 2–10 µL of the Transfer Solution from the well onto the region of interest of the FFPE tissue section mounted on a slide.
   b. Using the same 20-µL pipette tip, spread the Transfer Solution to ensure complete coverage of the region of interest, then scrape and break up the tissue with the pipette tip. The tissue should be a slurry of fine particles in the Transfer Solution.
3. Pipet the slurry from the slide back into the same well of the 96-well plate containing Transfer Solution.
4. Pipette the slurry up and down at least 5 times, leaving as much tissue as possible in the 96-well plate.
5. If needed, use the same tip to repeat steps 2–4, transferring as much of the region of interest as possible into the 96-well plate.

   Note: Although the final volume of Transfer Solution remaining in the 96-well plate can vary, no volumetric adjustment is required.
6. Add 21 µL of Direct Reagent to each well containing sample in the 96-well plate.

7. Set a pipette to 30 µL, then mix the Direct Reagent and slurry by pipetting up and down 10 times.

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

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

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

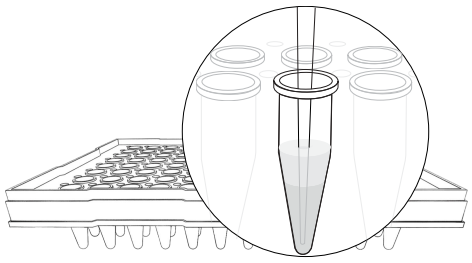
Temperature	Time
65°C	15 minutes
20°C	Hold (for up to 30 minutes)

Proceed to "Remove an aliquot for library preparation".

Note: See "(Optional) Remove deaminated bases from Direct FFPE DNA" on page 71 for an optional Uracil DNA Glycosylase (UDG) treatment procedure. Sample DNA concentration can be evaluated using an optional Qubit™ fluorometry protocol. See "(Optional) Qubit™ Fluorometer: Quantify the FFPE DNA" on page 72.

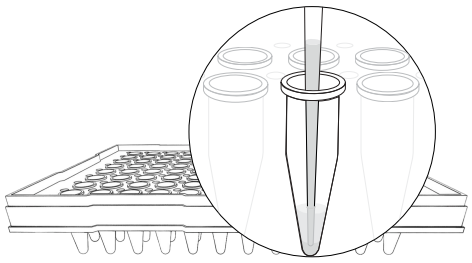
Remove an aliquot for library preparation

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.



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

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



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

   IMPORTANT! Avoid pipetting the upper phase that contains the Transfer Solution.

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

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

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

Set up DNA target amplification reactions

Set up DNA target amplification reactions using one of the following procedures for 1 and 2 primer pool panels. 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 65.

If you are using purified FFPE DNA, we recommend removing 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 purified FFPE DNA" on page 71.

Prepare DNA target amplification reactions—single primer pool

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

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

Component	Volume
2X 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. Seal the plate with a MicroAmp™ Clear Adhesive Film.

   Note: 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.

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

Proceed to "Amplify the targets" on page 27.

Prepare DNA target amplification reactions — 2 primer pools

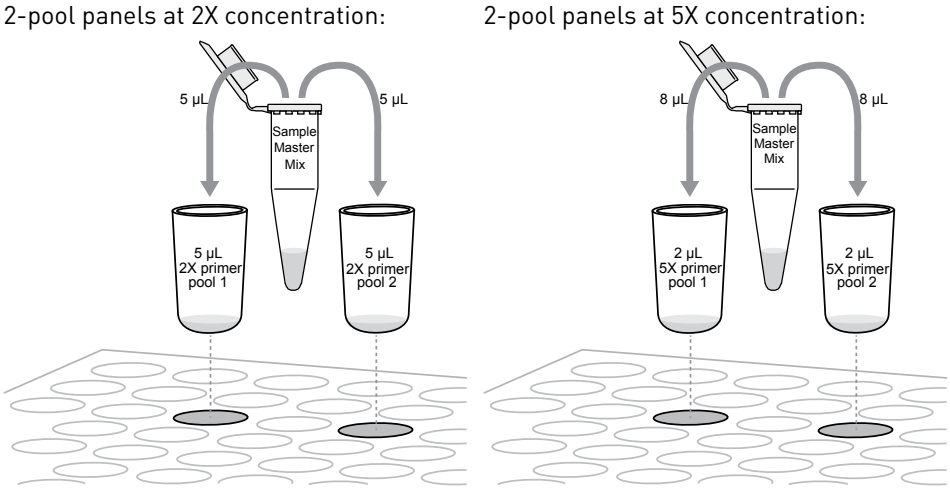
If you are using a DNA panel with 2 primer pools, set up two 10-µL amplification reactions, then combine them after target amplification to give a volume of 20 µL.

1. For 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.

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

Component	Volume
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.



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

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

   Note: 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™ Compression Pad on the plate, then load the plate into the thermal cycler.

Proceed to "Amplify the targets".

Amplify the targets

To amplify target regions, run the following program.

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

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

Cycle number recommendations in the preceding table are based on 10-ng DNA input. Adjust 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:

- Cycle number can be increased when input material quality or quantity is questionable.
- If two 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 roughly equivalent so that the selected cycle number for target amplification is optimal for all the samples in the run.

Exceptions to the amplification parameters recommended in the parameter table

Ion AmpliSeq™ panel	Primer pairs/pool	Description of change
Ion AmpliSeq™ Comprehensive Cancer Panel (Cat. No. 4477685)	~4,000	Use 8 minute 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

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 centrifuge briefly to collect the contents at the bottom of the wells.
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 a cold block, then quickly proceed to incubation.

1. One-primer pool panel: tap the plate gently on a hard flat surface, or centrifuge briefly to collect the contents at the bottom of the wells, 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 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.

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

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

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

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

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

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

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

Ion Xpress™ adapters only: Combine and dilute adapters

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

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

[1] X = barcode chosen

Note: Store diluted adapters at –20°C.

Perform the ligation reaction

1. If there is visible precipitate in the Switch Solution or the tube cap after thawing, vortex or pipet up and down at room temperature to resuspend before pipetting.
2. Briefly centrifuge the plate to collect the contents.
3. Carefully remove the plate seal, then add the following components in the order 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™ 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™ 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! Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.

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

1. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
2. Carefully remove the plate seal, then add 45 µL (1.5X sample volume) of Agencourt™ AMPure™ XP Reagent to each library. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.

   Note: 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.

   Note: If your magnet does not have two positions for shifting the beads, remove the plate from the magnet and gently pipet up and down 5 times (with the pipettor set at 100 µL), then return the plate to the magnet and incubate for 2 minutes or until the solution clears.
6. Repeat step 6 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 and 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 4, “Equalize the library“).
- Option 2: Quantify the library by qPCR (see Chapter 5, “Quantify the library by qPCR“).
- Option 3: Quantify the amplified library with a Qubit™ Fluorometer, or with the Agilent™ 2100 Bioanalyzer™ instrument (see Chapter 6, “Quantify the amplified library with a Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ instrument“).

3 Prepare Ion AmpliSeq™ RNA libraries

- Procedural guidelines 32
- Before each use of the kit 32
- Guidelines for RNA isolation, quantification, and input 33
- Reverse transcribe RNA 33
- Prepare cDNA target amplification reactions—single primer pool 34
- Prepare cDNA target amplification reactions—2 primer pools 35
- Amplify the targets 36
- Combine target amplification reactions (Only for RNA libraries with 2 primer pools) 37
- Partially digest amplicons 37
- Ligate adapters to the amplicons and purify 38

Procedural guidelines

- Minimize freeze-thaw cycles of Ion AmpliSeq™ panels by aliquoting as needed for your experiments. Panels can be stored at 4°C for one year.
- Use good laboratory practices to minimize cross-contamination of products. If possible, perform PCR setup in an area or room that is free of amplicon contamination. Always change pipette tips between samples.
- Use a calibrated thermal cycler specified in "Required materials not supplied" on page 15.
- 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.

Before each use of the kit

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

Guidelines for RNA isolation, quantification, and input

- See "Recommended materials and equipment" on page 16 for kits recommended for isolating total RNA.
- We recommend the Qubit™ RNA HS Assay Kit (Cat. No. Q32855) for quantifying RNA.
- Each reverse transcription reaction requires 1–100 ng of DNase-treated total RNA (≥0.14 ng/µL).
- In general, library yield from high quality RNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.
- Increasing the amount of RNA will usually result in higher quality libraries, especially when RNA quality or quantity is unknown. We recommend using 1 ng total RNA only with high-quality, well-quantified samples.

Reverse transcribe RNA

If you are starting from RNA, you must first reverse transcribe to cDNA. We recommend using the SuperScript™ IV VILO™ Master Mix (Cat. No. 11756050; ordered separately), which simplifies reaction setup and provides improved performance.

Note: If you are using the SuperScript™ VILO™ cDNA Synthesis Kit (Cat. No. 11754050) for reverse transcription, see "Reverse transcribe RNA with the SuperScript™ VILO™ cDNA Synthesis Kit" on page 70 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
SuperScript™ IV VILO™ Master Mix	2 µL
Total RNA (1–100 ng)	⩽ 8µL
Nuclease-free Water	to 10 µL
Total volume per well	**10 µL**

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

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

Temperature	Time
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 reactions are at the bottom of the wells, or if possible, centrifuge the plate to collect any droplets. Proceed to the next step:
   - If you are using an RNA panel with one primer pool, proceed to "Prepare cDNA target amplification reactions — single primer pool"
   - If you are using an RNA panel with two primer pools, proceed to “Prepare cDNA target amplification reactions—2 primer pools“

Prepare cDNA target amplification reactions—single primer pool

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

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

1. For RNA panels with one pool, remove the seal from the plate, 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™ Compression Pad on the plate, then place the sealed plate in a thermal cycler .

   Note: 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 36.

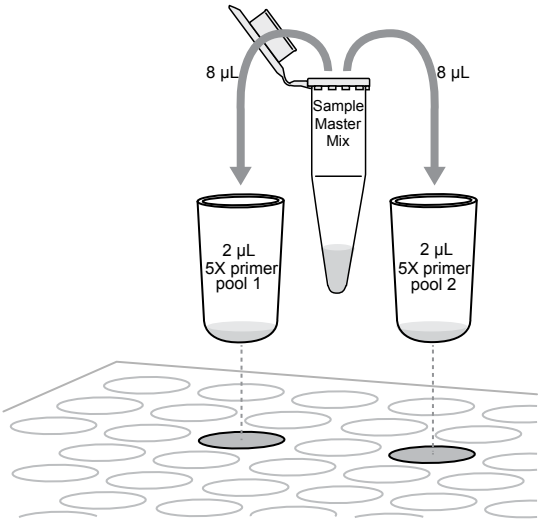
Prepare cDNA target amplification reactions—2 primer pools

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

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

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

2. Mix thoroughly by pipetting up and down 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™ Compression Pad on the plate, then place the sealed plate in a thermal cycler.

   Note: 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 36.

Amplify the targets

To amplify target regions, run the following program.

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

Primer pairs per pool (excluding gene fusion primer pairs)[1]	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

[1] 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.

Cycle number recommendations in the preceding table are based on 10-ng RNA input. Adjust 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:

- Cycle number can be increased when input material quality or quantity is questionable.

- If two 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 roughly equivalent so that the selected cycle number for target amplification is optimal for all the samples in the run.

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

Combine target amplification reactions (Only for RNA libraries with 2 primer pools)

1. Tap the plate gently on a hard flat surface, or centrifuge briefly to collect the contents at the bottom of the wells.
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 a cold block, then quickly proceed to incubation.

1. One-primer pool panel: tap the plate gently on a hard flat surface, or centrifuge briefly to collect the contents at the bottom of the wells, 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 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.
4. Place a compression pad on the plate, load in the thermal cycler, then run the following program:

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

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

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

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

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

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

Ion Xpress™ adapters only: Combine and dilute adapters

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

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

[1] X = barcode chosen

Note: Store diluted adapters at –20°C.

Perform the ligation reaction

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

3. Carefully remove the plate seal, then add the following components in the order 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™ 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™ 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! Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.

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

1. If possible, briefly centrifuge the plate to collect the contents in the bottom of the wells.

2. Carefully remove the plate seal, then add 45 µL (1.5X sample volume) of Agencourt™ AMPure™ XP Reagent to each library. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.

   Note: 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.

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

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 1–5 minutes. Do not overdry.

   IMPORTANT! Residual ethanol drops inhibit library amplification. If needed, centrifuge the plate and remove remaining ethanol before air-drying the beads. 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 4, "Equalize the library").
- Option 2: Quantify the library by qPCR (see Chapter 5, "Quantify the library by qPCR").
- Option 3: Quantify the amplified library with a Qubit™ Fluorometer, or with the Agilent™ 2100 Bioanalyzer™ instrument (see Chapter 6, "Quantify the amplified library with a Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ instrument").

4 Equalize the library

- Before you begin 41
- Amplify the library 42
- Wash the Equalizer™ Beads (if not previously performed) 43
- Add Equalizer™ Capture to the amplified library 43
- (Optional) Combine captured libraries 43
- Add Equalizer™ Beads and wash 44
- Elute the Equalized library 45
- (Optional) Combine Equalized libraries 45
- Store libraries 45

IMPORTANT!

- We recommend starting with the qPCR library quantification method (see Chapter 5, "Quantify the library by qPCR"). Alternatively, Qubit™ and Bioanalyzer™ methods can be used (Chapter 6, "Quantify the amplified library with a Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ instrument"). 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. If sample quality or quantity is variable or unknown (such as RNA from FFPE tissue, or Direct FFPE DNA), the qPCR and Qubit™/Bioanalyzer™ quantification methods can provide a higher success rate in terms of library yield and resulting number of sequencing reads.
- Standard library amplification parameters are not compatible with the Ion Library Equalizer™ Kit.

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

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

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 Platinum™ PCR SuperMix HiFi** (black cap) and **2 µL of Equalizer™ Primers** (pink cap) to each bead pellet. The Platinum™ PCR SuperMix HiFi and primers can be combined before addition.

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

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

   Note: The concentration of the amplified library can be confirmed by removing 2 µL of the reaction and evaluating with the Ion Library TaqMan® Quantitation Kit. The Ion Library Equalizer™ Kit should only be used when library concentrations are routinely >4,000 pM after library amplification.

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

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

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

2. For each reaction, pipet 3 µL of beads into a clean 1.5-mL tube, then add 6 µL/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 to each library amplification reaction.

   Note: The final equalized library concentration is dependent upon accurate pipetting of the Equalizer™ Capture reagent.

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. 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 77or "Ion Chip capacities for Ion AmpliSeq™ RNA libraries" on page 78).

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

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

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

Add Equalizer™ Beads and wash

1. Mix the washed Equalizer™ Beads by gentle vortexing or pipetting up and down.
2. If needed, carefully remove the seal from the plate, then **add 6 µL of washed Equalizer™ Beads** to each plate well containing the captured library (either combined or individual).
3. Set the pipette volume to 40 µL, then pipet the mixture up and down at least 5 times to mix thoroughly.
4. Incubate at room temperature for 5 minutes.

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

   Note: For uncombined libraries, 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.

   Note: 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.

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

Elute the Equalized library

1. Remove the plate from the magnet, then **add 100 µL of Equalizer™ Elution Buffer** to each pellet.
2. Seal the plate with MicroAmp™ 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.

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

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

(Optional) Combine Equalized libraries

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

Store libraries

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

5 Quantify the library by qPCR

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

Elute the library

1. Remove the plate 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 droplets. 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, containing the library, then combine with 198 µL of Nuclease-free Water.

Quantify library by qPCR and calculate the dilution factor

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

1. Prepare three 10-fold serial dilutions of the *E. coli* DH10B 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. 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. Select one of the programs in the following table. The fast cycling program was developed using the StepOnePlus™ System in fast run 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.

Reaction plate format	Stage	Temperature	Time
Fast run mode			
48- / 96-well Fast OR 384-well Standard	Hold (UDG incubation)	50°C	2 min
	Hold (polymerase activation)	95°C	20 sec
	Cycle (40 cycles)	95°C	1 sec
		60°C	20 sec
Standard run mode			
96-well Standard	Hold (UDG incubation)	50°C	2 min
	Hold (polymerase activation)	95°C	2 min
	Cycle (40 cycles)	95°C	15 sec
		60°C	1 min

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

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

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

   Note: A library that yields less than 100 pM can be rescued with library amplification. Combine 25 µL of unamplified library with 72 µL of Platinum™ PCR SuperMix HiFi and 3 µL of Library Amplification Primer Mix. Perform 5–10 library amplification cycles (see step 4 of "Amplify the library" on page 42 or "Amplify the library" on page 49 or for cycling conditions).

(Optional) Combine amplicon libraries

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

Store libraries

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

6 Quantify the amplified library with a Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ instrument

- Amplify the library 49
- Purify the amplified library 50
- Qubit™ Fluorometer: Quantify the library and calculate the dilution factor . . . 52
- Agilent™ 2100 Bioanalyzer™ instrument: Quantify the library and calculate the dilution factor 53
- (Optional) Combine amplicon libraries 53
- Store libraries 53

Ion AmpliSeq™ libraries must be amplified before quantification to enrich amplifiable material and obtain sufficient material for accurate quantification. Amplify the library using Platinum™ PCR SuperMix HiFi, 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 Platinum™ PCR SuperMix HiFi** and **2 µL of Library Amplification Primer Mix** to each bead pellet.

   Note: The Platinum™ PCR SuperMix HiFi and Library Amplification Primer Mix and can be combined before addition.

2. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge briefly to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 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™ Adhesive Film, place a MicroAmp™ Compression Pad on the plate, load in the thermal cycler, then run the following program:

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

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

Purify the amplified library

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

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

IMPORTANT!
- Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.
- Use freshly prepared 70% ethanol for the next steps. Combine 230 µL of ethanol with 100 µL of Nuclease-free Water per sample.
- Do **NOT** substitute a Dynabeads™-based purification reagent for the Agencourt™ Agencourt™ AMPure™ XP Reagent.

First-round purification

1. Tap the plate gently on a hard flat surface, or centrifuge briefly to collect the contents at the bottom of the wells, then remove the plate seal.
2. **Add 25 µL (0.5X sample volume) of Agencourt™ AMPure™ XP Reagent** 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 above, **add 60 µL (1.2X original sample volume) of Agencourt™ AMPure™ XP Reagent**. 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.

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

5. Repeat step 4 for a second wash.
6. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 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™ Adhesive Film, vortex thoroughly, then centrifuge to collect droplets. 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 (avg. 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 below.

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 manufacturer's instructions to perform a region analysis (smear analysis). Briefly:
   a. Select the **Data** icon in the Contexts panel, then view the electropherogram of the sample to be quantified.
   b. Select the **Region Table** tab below, then create a region spanning the desired amplicon peaks. Correct the baseline if needed.
   c. The molarity is automatically calculated and displayed in the table in pmol/L (pM).
2. Based on the calculated library concentration, determine the dilution that results in a concentration of ~100 pM.
   For example:
   - The library concentration is 3,000 pM.
   - The dilution factor is 3,000 pM/100 pM = 30.
   - Therefore, 10 µL of library mixed with 290 µL of Low TE (1:30 dilution) yields approximately 100 pM.
3. Dilute library to ~100 pM, 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".

Store libraries

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

A Tips and troubleshooting

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™ Side Magnet.
- If you observe evaporation in target amplification reactions, avoid using outside wells.
- Plate seals can be firmly applied using the applicator in 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 or IonCode™ adapters to avoid handling and diluting adapters. Alternatively, combine and dilute Ion Xpress™ adapters in large batches, then carefully aliquot into 96-well plates.
- If you are performing qPCR quantification, library amplification is unnecessary and the tube of Platinum™ PCR SuperMix High Fidelity supplied in the kit can be used for other applications. See the *Platinum™ PCR SuperMix High Fidelity User Guide* for instructions.
- When using the Qubit™ 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 roughly equivalent so that the selected cycle number is optimal for all the samples in the run.
- If you combine aliquots of captured libraries before adding Equalizer™ Beads, save the unused portions of 9-cycle amplified libraries for repeat analysis if needed.
- When trying the Ion Library Equalizer™ Kit for the first time, quantify the amplified libraries by qPCR to assure that libraries are routinely >4 nM in concentration.
- When setting up sample-specific master mixes for panels with 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.
- Shorter anneal/extend times can work well with some panels. When testing shorter times, look for decreased representation of AT-rich amplicons.
- During target amplification, 98°C can be used for the enzyme activation and denaturation steps for some panels, especially those targeting cDNA. This change can improve underperforming amplicons.

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 AMPure™ XP Beads. However, any remaining ethanol inhibits the library amplification reaction.
- When using qPCR quantification, the purification step after adapter ligation ("Purify the unamplified library") can be eliminated. Two additional target amplification cycles help reduce the amount of relative by-products that can go into template preparation.
- 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 amplified 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 25. Adjust target amplification cycle number and anneal/extend time accordingly for the 2 new pools (~8,000 primer pairs/pool).

  Note: 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 (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 21).
    b. Incubate for 5 minutes at room temperature to lyse cells.
    c. Add 1 µL of Stop Solution (from the Ambion™ Cell Lysis Kit) without pipetting up and down, then incubate for 2 minutes.
    d. Proceed to target amplification by adding 4 µL 5X Ion AmpliSeq™ HiFi Mix and 2X or 5X primer pool for a total volume of 20 µL.

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

Troubleshooting

Ion AmpliSeq™ Direct FFPE DNA Kit

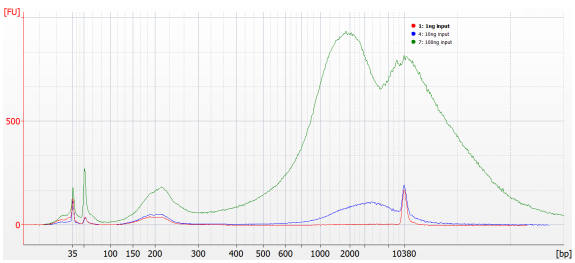
Observation	Possible cause	Recommended action
Tissue does not transfer from slide	Not enough Transfer Solution.	Hold the slide at a 45° angle and pipet extra Transfer Solution to the top of the slide allowing the tissue to flow towards the bottom. Remove collected tissue from the bottom, repeat as needed.
	Tissue is clumpy.	Transfer the mass of tissue to a collection tube, then continue breaking it up with a pipet tip.
		Pre-incubate with Transfer Solution on slide for 5 minutes, then proceed to scraping.
Difficulty scraping tissue off the slide	Tissue is fibrous.	Scrape with 200-µL tip prior to transfer, using a circular motion, then continue with a 20-µL tip.
		Scrape and homogenize tissue with 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 sample may still be cloudy, this will not affect performance. Ensure homogenous mixing of the sample prior to removing an aliquot for target amplification. Undissolved tissue that can be aspirated with a pipet tip may still be added to the Target Amplification reaction.
		Centrifuge at ≥1,000 × *g* for 1 minute, then transfer 15 µL to a fresh tube, avoiding the fibrous pellet.
Transfer Solution and Direct Reagent do not separate into two phases	Too much paraffin in sample.	Use a scalpel blade to scrape away undesired paraffin prior to adding Transfer Solution to the desired tissue area.
		Centrifuge at ≥1,000 × *g* for 1 minute, then transfer 15 µL to a fresh tube, avoiding the fibrous pellet and tube walls.

Observation	Possible cause	Recommended action
Transfer Solution and Direct Reagent do not separate into two phases		Perform partial deparaffinization before adding Transfer Solution to tissue on the slide: 1. Submerge slide in 100% xylene for 30 seconds. 2. Remove the slide, then drain any excess xylene. 3. Submerge 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 pipet tip.	Return tip contents to reaction tube, then centrifuge at ≥1,000 × *g* for 1 minute to separate phases. Move pipet quickly through the upper phase when transferring. **Note:** Transfer Solution will not interfere with target amplification.
Low AmpliSeq™ library concentration (see "Library yield and quantification" on page 58 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 AmpliSeq™ library. Re-prepare 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) Samples with low DNA yield can still be sufficient to generate an AmpliSeq™ library.	FFPE tissue has low DNA content.	Increase the number of target amplification cycles by 2 or 3.

Library yield and quantification

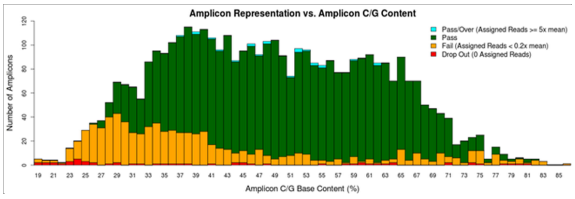
Observation	Possible cause	Recommended action
Library concentration is low—general (Library concentration is NOT indicative of quality.)	Sample DNA or RNA was mis-quantified.	Requantify sample DNA using the TaqMan® RNase P Detection Reagents Kit; quantify RNA with a Qubit™ Fluorometer.

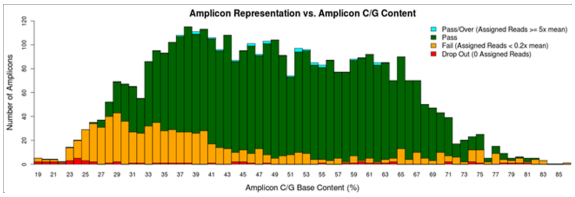
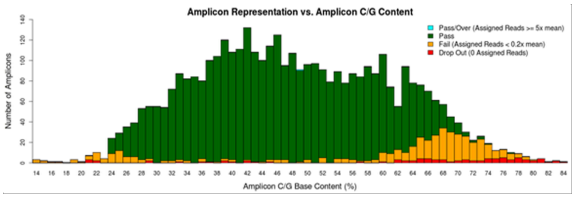
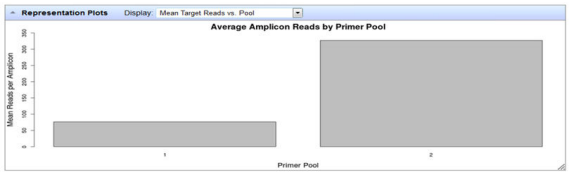
Observation	Possible cause	Recommended action
Library concentration is low—general (Library concentration is NOT indicative of quality.)	Residual ethanol in sample DNA or RNA inhibited target amplification.	Incubate uncapped tube in hood for 1 hour.
		Speed-vac tube at room temperature for 5 minutes.
	Residual ethanol from AMPure™ purification inhibited library amplification.	Carefully remove all drops of ethanol before library amplification, then centrifuge plate, if necessary.
	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.	Be sure to save the supernatant during first-round purification, and save the bead pellet during the second round.
	AMPure™ XP Beads were over-dried.	Do not dry the AMPure™ XP Beads more than 5 minutes.
	AMPure™ XP Beads inhibited library amplification.	Transfer library off of beads prior to amplification.
	qPCR cycling time is too short.	Use standard qPCR cycling for library designs >175 bp instead of Fast cycling.
	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 prior to equalization was inadequate.	Use the Ion Library Equalizer™ Kit only when library yield is consistently above the minimum expected concentration listed in this user guide. This can be assessed with qPCR, by removing 2 µL after library amplification.
	Equalizer™ Beads were not washed.	Be sure to wash Equalizer™ Beads before use.
	Wrong library amplification primers were used.	Use the Equalizer™ Primers provided in the Ion Library Equalizer™ Kit.
	Residual Equalizer™ Wash Buffer was present after wash.	Carefully remove all of the Equalizer™ Wash Buffer before elution.

Observation	Possible cause	Recommended action
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 mis-assigned.	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 AMPure™ XP Reagent volume from 25 µL (0.5X) to 35 µL (0.7X) in the first-round purification.
	Inserts are concatamerizing during the ligation step.	Reduce nucleic acid input amount.
		Requantify sample(s) with a Qubit™ Fluorometer.
		Reduce target amplification cycle number.

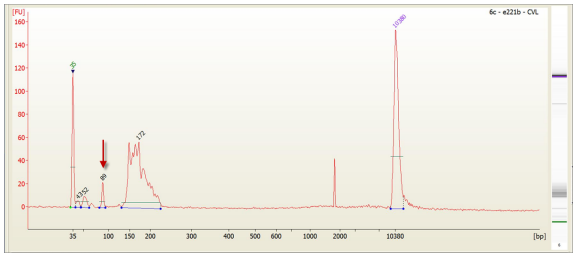
Low amplicon uniformity (DNA only)

Observation	Possible cause	Recommended action
Short amplicons are under-represented	Purification was poor.	Vortex AMPure™ XP Reagent thoroughly before use, and be sure to dispense the full volume.
		100% ethanol is difficult to pipet accurately; it is essential to pre-wet pipette tips.
		In post-ligation library purification, increase AMPure™ XP Reagent volume from 45 µL (1.5X) to 50 µL (1.7X).
		In amplified library purification, increase AMPure™ XP Reagent volume in second round from 60 µL (1.2X) to 70 µL (1.4X).

Observation	Possible cause	Recommended action
Short amplicons are under-represented	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 275 bp and can require 650 flows to achieve end-to-end reads, if needed. Determine amplicon length from the panel BED file.
	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.
AT-rich amplicons are under-represented  **Example of loss of AT-rich amplicons.** Within the Coverage Analysis Plugin, amplicon representation is plotted by GC content for an Ion AmpliSeq™ Panel. Amplicons with 23-50% GC show an excess failure rate (less than 20% of the mean read depth).	Target amplification was inefficient.	Double the anneal/extend time in the target amplification reaction.
		Decrease the anneal/extend temperature of the target amplification reaction from 60°C to 58°C.
		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.

Observation	Possible cause	Recommended action
AT-rich amplicons are under-represented  **Example of loss of AT-rich amplicons.** Within the Coverage Analysis Plugin, amplicon representation is plotted by GC content for an Ion AmpliSeq™ Panel. Amplicons with 23-50% GC show an excess failure rate (less than 20% of the mean read depth).	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 Coverage Analysis Plugin, amplicon representation is plotted by GC content for an Ion AmpliSeq™ Panel. Amplicons with 60-80% GC show an excess failure rate (less than 20% of the mean read depth).	Denaturation was inadequate during target amplification.	Use a calibrated thermal cycler.
	Target amplification was inefficient.	Increase the anneal/extend temperature of the target amplification reaction from 60°C to 62°C for the first two cycles of the target amplification reaction
	Library amplification was inefficient.	Do not amplify the library (not required for qPCR quantification).
	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  **Example of pool imbalance.** Within the Coverage Analysis 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.
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 AMPure™ XP Reagent volume from 45 µL (1.5X) to 30 µL (1X).
		In amplified library purification, decrease AMPure™ XP Reagent volume in second round from 60 µL (1.2X) to 50 µL (1.0X).
	Adapter dimers formed during reaction setup or during digestion.	Do not combine Adapters, DNA Ligase, and Switch Solution prior to addition.
		Use a 65°C temperature incubation instead of 60°C during the amplicon digestion step.
	Adapter concentration was too high.	Ensure that barcode adapters are diluted properly.
The number of on-target reads is lower than expected	Unknown.	Increase the number of target amplification cycles by two, or increase the anneal/extend temperature of the target amplification reaction from 60°C to 62°C 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%.
	Library was mis-quantified.	Ensure that library was quantified accurately.

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

- Prepare DNA target amplification reactions for panels with 3 or 4 primer pools 65
- Reverse transcribe RNA with the SuperScript™ VILO™ cDNA Synthesis Kit . 70
- (Optional) Remove deaminated bases from Direct FFPE DNA 71
- (Optional) Remove deaminated bases from purified FFPE DNA 71
- (Optional) Qubit™ Fluorometer: Quantify the FFPE DNA 72

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.

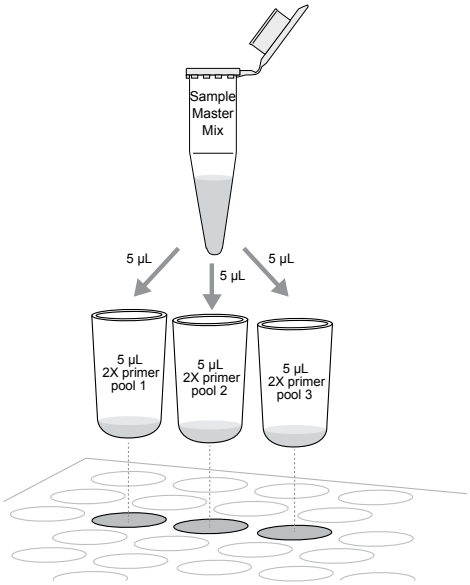
1. For panels with 3 primer pools, use the following table to prepare for each sample a target amplification master mix without primers in a 1.5-mL tube.

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

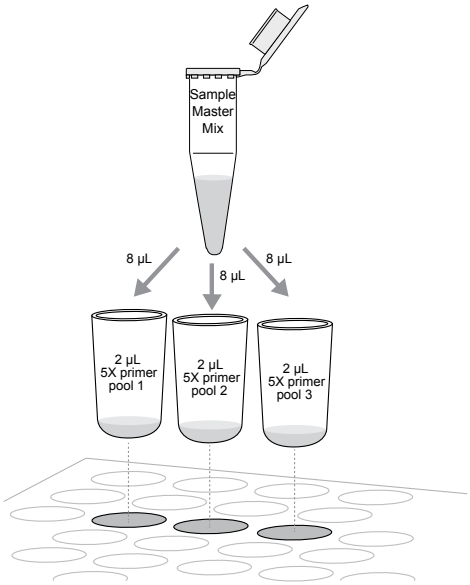
Component	Volume
3-pool panels at 2X concentration	
5X Ion AmpliSeq™ HiFi Mix (red cap)	7 µL
DNA (3–100 ng), or direct FFPE DNA preparation	≤10.5 µL
Nuclease-free Water	to 17.5 µL
3-pool panels at 5X concentration	
5X Ion AmpliSeq™ HiFi Mix (red cap)	7 µL
DNA (3–100 ng), or direct FFPE DNA preparation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



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

3. Seal the plate with a MicroAmp™ Adhesive Film, then place a MicroAmp™ Compression Pad on the plate. Proceed to "Amplify the targets" on page 27. See the following instructions for modifications to the protocol after target amplification when you use a 3-pool panel.

   Note: 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

Target amplification reactions need to be combined after amplification before proceeding to the digestion, adapter ligation, and purification steps.

1. After amplification, centrifuge briefly to collect droplets, 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.

   Note: For 3-pool panels, increase the volumes of FuPa Reagent, ligation components, and AMPure™ XP Reagent volumes in the next steps by 50%:

Primer pools	FuPa Reagent	Switch Solution	Barcode Adapters	DNA Ligase	AMPure™ XP Reagent
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.

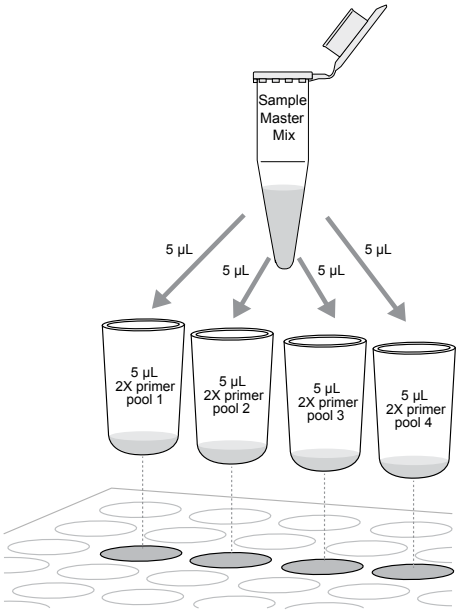
1. For panels with 4 primer pools, use the following table to prepare for each sample a target amplification master mix without primers in a 1.5-mL tube.

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

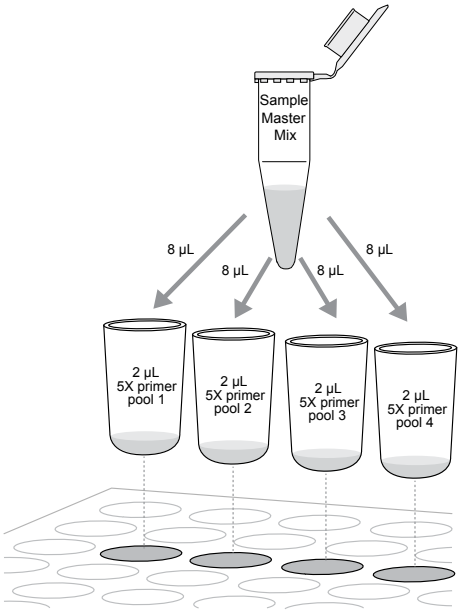
Component	Volume
4-pool panels at 2X concentration	
5X Ion AmpliSeq™ HiFi Mix (red cap)	9 µL
DNA (4–100 ng), or direct FFPE DNA preparation	≤13.5 µL
Nuclease-free Water	to 22.5 µL
4-pool panels at 5X concentration	
5X Ion AmpliSeq™ HiFi Mix (red cap)	9 µL
DNA (4–100 ng), or direct FFPE DNA preparation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



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

3. Seal the plate with a MicroAmp™ Adhesive Film, then place a MicroAmp™ Compression Pad on the plate. Proceed to "Amplify the targets" on page 27. See the following instructions for modifications to the protocol after target amplification when you use a 4-pool panel.

   Note: 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

Target amplification reactions need to be combined after amplification before proceeding to the digestion, adapter ligation, and purification steps.

1. After amplification, centrifuge to collect droplets, 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.

   Note: For 4-pool panels, double the volumes of FuPa Reagent, ligation components, and AMPure™ XP Reagent in the next steps:

Primer pools	FuPa Reagent	Switch Solution	Barcode Adapters	DNA Ligase	AMPure™ XP Reagent
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 (Cat. No. 11754050) to reverse transcribe RNA, use the following 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™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least five times before sealing the plate.

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

Temperature	Time
42°C	30 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, centrifuge the plate to collect any droplets. Proceed to the next step:

   - If you are using an RNA panel with one primer pool, proceed to "Prepare cDNA target amplification reactions—single primer pool" on page 34
   - If you are using an RNA panel with two primer pools, proceed to "Prepare cDNA target amplification reactions—2 primer pools" on page 35

(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 (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, centrifuge briefly to collect contents, then re-separate 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.

Proceed immediately to "Remove an aliquot for library preparation" on page 23.

(Optional) Remove deaminated bases from purified FFPE DNA

IMPORTANT! If using DNA isolated from sources other than FFPE tissue, proceed directly to "Set up DNA target amplification reactions" on page 24.

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 centrifuge briefly to collect the contents.

   Note: 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 centrifuge briefly 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 "Set up DNA target amplification reactions" on page 24. 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 appropriate to the panel you are using, adjusting the volume down to achieve the desired DNA input.

C

Strategies for combining Ion AmpliSeq™ libraries

- Combine libraries prepared with one panel for equal depth of coverage 73
- Combine libraries prepared from one panel to vary depth of coverage 74
- Combine DNA and RNA libraries to obtain different numbers of reads 75
- Combine libraries prepared using different panels for equal coverage 76
- Ion Chip capacities for Ion AmpliSeq™ DNA libraries sequenced at equal depths 77
- Ion Chip capacities for Ion AmpliSeq™ RNA libraries 78

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 77, and "Ion Chip capacities for Ion AmpliSeq™ RNA libraries" on page 78 for more information on the numbers of libraries that can be combined on a single chip.

Combine libraries prepared with one panel for equal depth of coverage

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

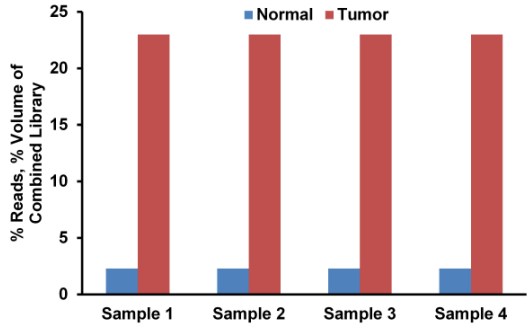
For example, if 16 libraries prepared with the same Ion AmpliSeq™ DNA or RNA panel are combined in a single templating and sequencing reaction:

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

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

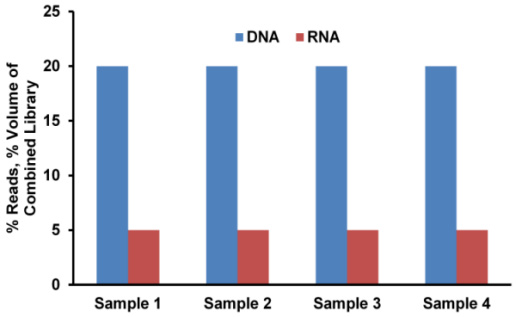
For the example above, if 8 libraries are prepared with the same Ion AmpliSeq™ panel:

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:

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:

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

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

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 required.

For a given chip, as the number of amplicons increases, the number of libraries that can be accommodated per sequencing run decreases. This relationship is shown in the following table. The numbers in the following table serve as a guide for approximate capacities. 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.

Chip	Ion 314™		Ion 316™ Ion 510™		Ion 318™ Ion 520™		Ion 530™		Ion PI™ Ion 540™		Ion 550™	
Average depth	**150X**	**2500X**	**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**	**95% >30X**	**95% >500X**
Number of amplicons per library	**Approximate number of libraries per chip**											
12–24	84	8	272	26	>384	48	>384	152	>384	>384	>384	>384
25–48	48	4	152	14	272	26	>384	84	>384	272	>384	>384
49–96	26	2	84	8	152	14	>384	48	>384	152	>384	243
97–192	14	1	48	4	84	8	272	26	>384	84	>384	134
193–384	8	—	26	2	48	4	152	14	>384	48	>384	77
385–768	4	—	14	1	26	2	84	8	272	26	>384	41
769–1,536	2	—	8	—	14	1	48	4	152	14	243	22
1,537–3,072	1	—	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

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 as most targets will not be present, and therefore will not create library molecules. For most fusion detection assays, only ~250,000 reads/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.

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

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

	Ion Chip						
	Ion 314™ Chip	**Ion 316™ Chip**	**Ion 510™ Chip**	**Ion 318™/ Ion 520™ Chip**	**Ion 530™ Chip**	**Ion PI™/ Ion 540™ Chip**	**Ion 550™ Chip**
Chip capacity in reads (M)	0.3–0.5	1–2	2–3	3–5	15–20	60–80	100–130

Example:

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

Sequencing depth desired = 5000 reads per amplicon

Number of primer pairs = 100

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

D

Ion AmpliSeq™ Made-to-Order Panels

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 primer. You can use the plates to:

- 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

Primer pairs from different panels can be combined as long as the amplicons within a pool do not overlap. When a panel with a new footprint is created, the designed BED file should be modified accordingly.

Prepare primer pools from plates

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

- For panels with up to 96 primer pairs per pool, combine and dilute to 1,000 nM per primer (5X, 1,000 nM).
- For panels with 97–1,228 primer pairs per pool, combine and dilute to 250 nM per primer (5X, 250 nM).
- For panels with 1,229–3,072 primer pairs per pool, combine and dilute to 100 nM per primer (2X, 100 nM).

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

1. Vortex the sealed primer plate, then centrifuge briefly to collect 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
5X, 1,000 nM (12–96 primer pairs)	Add Low TE to a final volume of 0.614 mL[1].
5X, 250 nM (97–1,228 primer pairs)	Add Low TE to a final volume of 2.456 mL [2].
2X, 100 nM (1,229–3,072 primer pairs)	Add Low TE to a final volume of 6.140 mL.
50X spike-in panel, 2,500 nM (1–123 primer pairs)	Add Low TE to a final volume of 0.246 mL.

[1] Can be prepared at 2X, 400 nM by increasing the final volume to 1.536 mL.
[2] Can be prepared at 2X, 100 nM by increasing the final volume to 6.140 mL.

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

E Expand any Ion AmpliSeq™ panel by adding a spike-in panel

An Ion AmpliSeq™ On-Demand Panel can be modified by using a spike-in panel. Spike-in panels enable addition of new content, control assays, and improvements in coverage uniformity (for example, by adding the same primer pair to two or more primer pools).

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

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

Number of primer pairs/pool			Target Concentration (Combined)	Instructions
Primary Panel	Spike-in	Combined		
Chef-Ready primary panels				
Add 6.4 µLof 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 µLof 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 centrifuge briefly to collect 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/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

F

Ion AmpliSeq™ Sample ID Panel

Using the Ion AmpliSeq™ Sample ID Panel

The Ion AmpliSeq™ Sample ID Panel is an Ion AmpliSeq™ Spike-in panel that is used to add amplicons to create a unique identifier for human gDNA samples. It is comprised of 9 primer pairs at a 20X concentration (1000 nM per primer). If you are using a Ready-to-Use, Made-to-Order, Community, or On-Demand Panel in a manual Ion AmpliSeq™ protocol, use the following procedure to create a sample signature in your panel.

1. For addition to a primary panel with greater than 96 primer pairs per pool, add 1 µL of the Ion AmpliSeq™ Sample ID Panel to a 20 µL target amplification reaction.

   Note:
   - For addition to a primary panel with less than 96 primer pairs per pool, optimization may be required to obtain the desired read coverage distribution of Sample ID amplicons relative to primary panel amplicons.
   - If a primary panel has more than one primer pool, the Ion AmpliSeq™ Sample ID Panel can be added to one or all primer pools.
   - 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. Select the following settings when creating a new Planned Run in the Torrent Browser Planned Run Wizard:
   a. In the **Kits** step, select the **Control Sequence**, then choose **Ion AmpliSeq Sample ID panel** from the dropdown list.

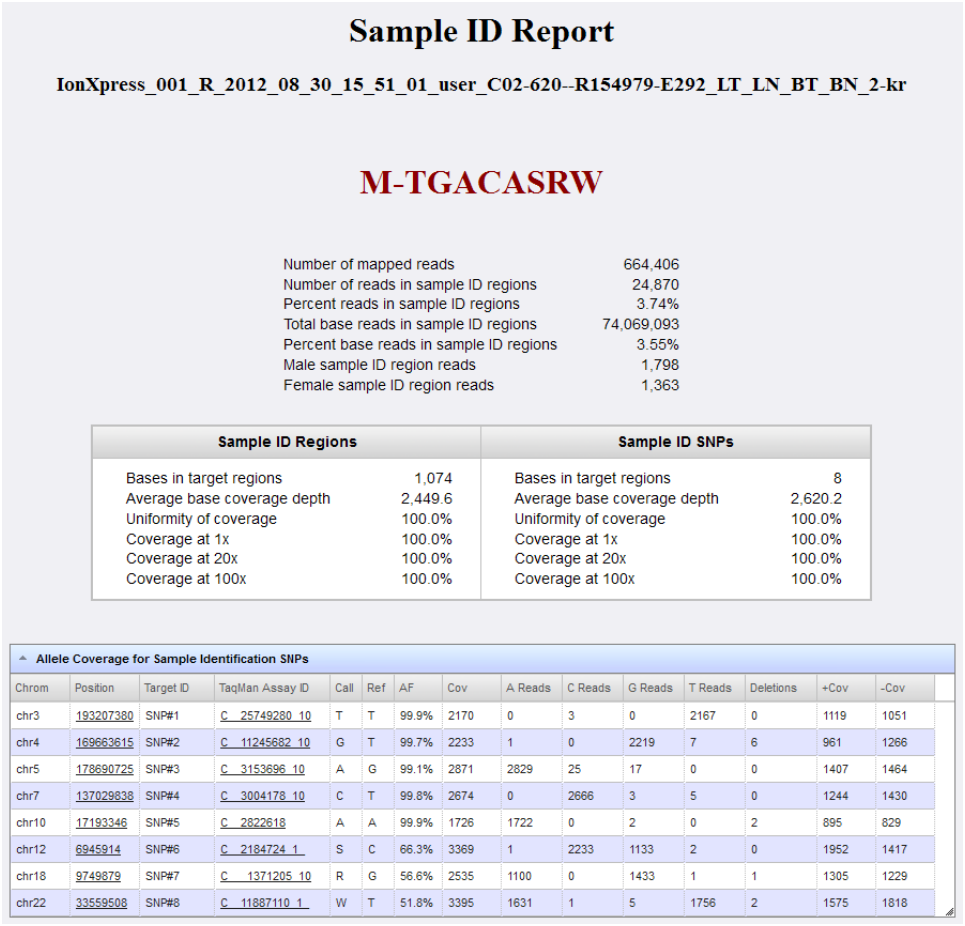
   b. In the **Plugins** step, select the **coverageAnalysis** plugin checkbox, then click **Configure** (see "coverageAnalysis plugin" on page 84).

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

      Note: Selecting the **Sample Tracking** checkbox enables the analysis to produce a statistic for reads mapped to Sample ID targets so that the level of off-target reads is accurately represented in the Coverage Analysis Report. If **Sample Tracking** is not selected, Sample ID reads are counted as off-target reads.

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

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

G Data analysis

For more information for setting up and performing data analysis with Torrent Suite™ Software, see *Torrent Suite™ Software Help* accessed from the Torrent Browser **Help** menu, or downloaded from **thermofisher.com** as a PDF.

See the *Ion Reporter™ Software Help* for more information for using the suite of bioinformatics tools in Ion Reporter™ Software to streamline variant analysis and reporting.

Details for the coverageAnalysis and ampliSeqRNA plugins are presented below.

coverageAnalysis plugin

Use the coverageAnalysis plugin to view statistics and graphs that describe the level of sequence coverage produced for targeted genomic regions. The results in the **Summary** screen for a run analyzed with the plugin vary based on the library type that you select when you configure the plugin. You can export some charts as graphics, such as the **Amplicon** and **Reference Coverage** charts.

coverageAnalysis plugin configuration

The coverageAnalysis plugin uses the following settings.

Setting	Description
The following settings are available for all library types.	
Reference Genome	The reference genome selected in the Planned Run.
Library Type	The default value is the library type selected in the Planned Run, and it can be changed only if the plugin is run manually. If you change the library type, a different report is generated.
Targeted Regions	The targeted regions are selected in the Planned Run, and can be changed only after the run is complete if the plugin is run manually. Target regions can be overwritten by the specific barcode targets. Select the targeted regions file from the list. For whole genome and Ion Total RNA-Seq sequencing runs, you typically select **None**.

Setting	Description
Barcode-specific Targets	This option is available only when the coverageAnalysis plugin is run manually. Select the checkbox to assign specific target region files to individual barcodes. 1. Select a specific barcode. 2. Select the specific Target Regions file to associate with the selected barcode. 3. Click **Add**. 4. Repeat steps 1 through 3 to associate additional barcodes with specific Target Region files. Alternatively, you can copy and paste the barcode/target file pairs manually. Barcodes without a Target Region specified above assume the default target specified by the **Target Regions** option. For targeted applications, any barcode targets specifically set to **None**, or defaulting to **Target Regions** set to **None**, are omitted from subsequent analysis. When the **Barcode-specific Targets** option is deselected, all barcodes use the targets specified by the Target Regions, even if barcode-specific targets are listed.
The following are advanced options.	
Minimum Aligned Length	Specify the minimum aligned length that is required to ensure that the read is included in an analysis.
M**inimum Mapping Quality**	Specify a minimum value that reads must exceed to be included in the analysis.
Tier 1 Coverage Depth	Specify the first-tier coverage depth at which percentage of target coverage is reported. This value must be at least 2, because the coverage depth output is always specified at 1*x* read depth. The default value of 20 means that the percentage of targets, total base targets, and/or individual target bases with at least 20 reads is reported.
Tier 2 Coverage Depth	Specify the second-tier coverage depth at which percentage of target coverage is reported. This value must be greater than the value used for the first-tier coverage. The default value of 100 means that the percentage of targets, total target bases, and/or individual target bases with at least 100 reads is reported.
Tier 3 Coverage Depth	Specify the third-tier coverage depth at which percentage of target coverage is reported. This value must be greater that the value used for the second-tier coverage. The default value of 500 means that the percentage of targets, total target bases, and/or individual target bases with at least 500 reads is reported.

Setting	Description
The following settings are available only with specific library types.	
Uniquely Mapped Reads	Select this option to analyze only reads that are mapped to a unique location in the reference. Reads that are non-uniquely mapped can have equally well-aligned reads that are mapped to multiple locations, and are typically mapped randomly to one.
Sample Tracking	The Ion AmpliSeq™ Sample ID Panel is a companion panel of 9 primer pairs that can be added to any Ion AmpliSeq™ human gDNA panel during target amplification to generate a unique identification tag for research samples. Select this checkbox if you added the Ion AmpliSeq™ Sample ID Panel to your library.
Target Padding	Enter a number to pad the target by the number of bases entered. If you do not enter a number, the default of 0 is used.
No**n-duplicate Reads**	Select the checkbox to avoid duplicates. The analysis must have included alignments with **Mark Duplicates** enabled.

Review coverageAnalysis plugin results

The coverageAnalysis plugin generates a Coverage Analysis Report. This report includes read statistics and several charts. The statistics and charts that are presented depend on the library type for the analysis.

The report lists the samples, the number of mapped reads, the percentage of valid reads, and the percentage of targets detected. A series of log2 RPM pair-correlation plots are included for rapid correlation analysis. Microsoft™ Excel™-compatible reports are also generated, including differential expression tables. Additional details regarding read coverage are also provided on a per-barcode basis, along with a list of gene annotations for each sequenced region.

You can download statistics files and the aligned reads BAM file from the file links at the bottom of the Coverage Analysis Report.

After the sequencing run completes, review the plugin results in the report summary.

1. In the **Data** tab, click **Completed Runs & Reports**.
2. In the list of runs, find the run of interest, then click the link in the **Report Name** column.
3. In the left navigation menu, click **coverageAnalysis** to view the plugin summary. A summary table of the coverage analysis, by barcode, is included in the **Summary** screen.
4. Click a link in the **Barcode Name** column of the summary table to open a detailed **Coverage Analysis Report** window for that barcoded sample. Alternatively, click the **coverageAnalysis.html** link to open the summary table for all barcodes in a new window.
5. Click the links at the bottom of the Coverage Analysis Report to download associated statistics and summary files for each barcoded sample in the run.

Reads statistics

The library type determines which statistics are presented. This table shows the statistics for an Ion AmpliSeq™ DNA report. Some of these statistics are not available for other library types or are replaced by alternative statistics. Definitions are in tooltips. Almost every statistic, plot, link, and functional widget in the report provides tooltips with definitions. Hover over a heading or description in the report to view the tooltip.

Statistic	Description
Number of mapped reads	The total number of reads mapped to the reference.
Number of reads on target	The total number of reads mapped to any targeted region of the reference. A read is on target if at least one aligned base overlaps a target region. A read that overlaps a targeted region but where only flanking sequence is aligned, for example, due to poor matching of 5' bases of the read, is not counted.
Target Base Coverage	Summary statistics for targeted base reads of the reference. A base covered by multiple target regions is counted only once per sequencing read.
Bases in target regions	The total number of bases in all specified target regions of the reference.
Percent of reads on target	The percentage of reads mapped to any targeted region relative to all reads mapped to the reference.
Total aligned base reads	The total number of bases covered by reads aligned to the reference.
Total base reads on target	The total number of target bases covered by any number of aligned reads.
Percent base reads on target	The percent of all bases covered by reads aligned to the reference that covered bases in target regions.
Bases in targeted reference	The total number of bases in all target regions of the reference.
Bases covered (at least 1x)	The total number of target bases that had at least one read aligned over the proximal sequence. Only the aligned parts of each read are considered. For example, unaligned (soft-cut) bases at the 5' ends of mapped reads are not considered. Covered target reference bases can include sample DNA read base mismatches, but does not include read base deletions in the read, nor insertions between reference bases.
Average base coverage depth	The average number of reads of all targeted reference bases.
Uniformity of base coverage	The percentage of bases in all targeted regions (or whole-genome) covered by at least 0.2x the average base coverage depth.

Statistic	Description
Average base read depth	The average number of reads of all targeted reference bases that were read at least one time.
Genome Base Coverage	Summary statistics for base reads of the reference genome.
Genome base coverage at *N* x	The percentage of reference genome bases covered by at least *N* reads.
Target coverage at *N* x	The percentage of target bases covered by at least *N* reads.
Targets with no strand bias	The percentage of all targets that did not show a bias toward forward or reverse strand read alignments. An individual target has read bias if it has at least 10 reads and the percentage of forward or reverse reads to total reads is greater than 70%.
Amplicon Read Coverage	Summary statistics for reads assigned to specific amplicons. Each sequence read is assigned to exactly one of the amplicons specified by the targets file. Reads are assigned to particular amplicon targets based if their (5') mapping location being sufficiently close to the end of the amplicon region, taking the read direction (mapping strand) into account.
Number of amplicons	The number of amplicons specified in the target regions file.
Percent assigned amplicon reads	The total number of reads that were assigned to individual amplicons. A read is assigned to a particular (inner) amplicon region if any aligned bases overlap that region. If a read might be associated with multiple amplicons this way it is assigned to the amplicon region that has the greatest overlap of aligned sequence.
Average reads per amplicon	The average number of reads assigned to amplicons.
Uniformity of amplicon coverage	The percentage of bases in all targeted regions (or whole-genome) covered by at least 0.2x the average base read depth.
Amplicons with at least *N* reads	The percentage of all amplicons that had at least *N* reads.
Amplicons with no strand bias	The percentage of all amplicons that did not show a bias towards forward or reverse strand read alignments. An individual amplicon has read bias if it has at least 10 reads and the percentage of forward or reverse reads to total reads is greater than 70%.

Statistic	Description
Amplicons reading end-to-end	The percentage of all amplicons that were considered to have a sufficient proportion of assigned reads (70%) that covered the whole amplicon target from 'end-to-end'. To allow for error, the effective ends of the amplicon region for read alignment are within 2 bases of the actual ends of the region.
Amplicon based composition bias	A number that represents the proportion of amplicons showing low representation (<.2x mean reads) in the lower and/or upper quartiles of amplicons ordered by increasing GC base pair content of their insert sequences. The value is relative to that in the center 50th percentile of amplicons and weighted by the standard deviation of representation over all amplicons.

Example statistics

The following is an example of the plugin statistics for an Ion AmpliSeq™ run.

Coverage Analysis Report

Sample Name: T3

IonXpress_006_R_2016_02_11_17_46_25_user_p04-574

Library type: AmpliSeq Exome
Reference: hg19 (DNA)
Target regions: AmpliSeqExome.20131001.designed



Number of mapped reads	40,285,453
Percent reads on target	94.86%
Average base coverage depth	110.0
Uniformity of base coverage	92.05%

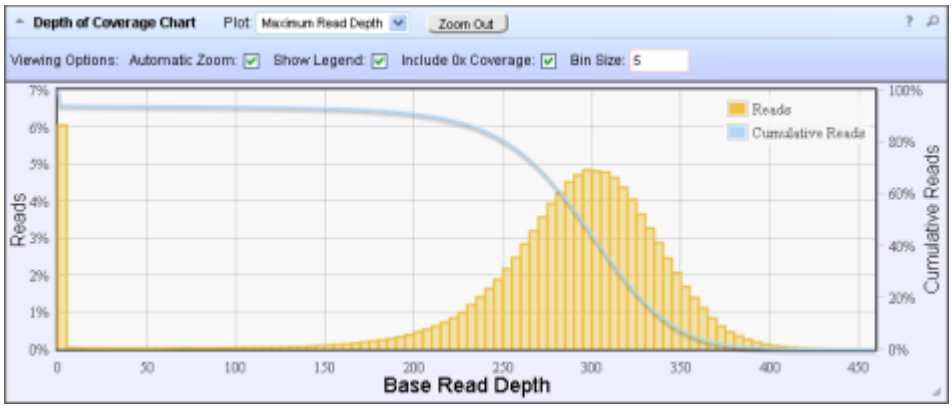
Amplicon Read Coverage		Target Base Coverage	
Number of amplicons	293,903	Bases in target regions	57,742,646
Percent assigned amplicon reads	94.86%	Percent base reads on target	94.86%
Average reads per amplicon	130.0	Average base coverage depth	110.0
Uniformity of amplicon coverage	93.33%	Uniformity of base coverage	92.05%
Amplicons with at least 1 read	99.50%	Target base coverage at 1x	99.20%
Amplicons with at least 20 reads	95.21%	Target base coverage at 20x	92.92%
Amplicons with at least 100 reads	56.84%	Target base coverage at 100x	46.00%
Amplicons with at least 500 reads	0.30%	Target base coverage at 500x	0.19%
Amplicons with no strand bias	90.41%	Target bases with no strand bias	76.69%
Amplicons reading end-to-end	19.19%	Percent end-to-end reads	51.22%

The Reference Coverage chart is an overlay of where target regions are defined and overlap on the reference.

Example charts

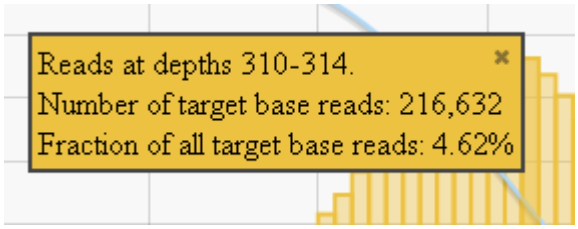
Many of the charts that are generated by the coverageAnalysis plugin include a **Plot** menu that allows you to change characteristics of the chart. For example, you can show both strands.

The button (in the top right corner of a chart) opens the chart **Viewing Options** panel. The button opens a description of the chart.

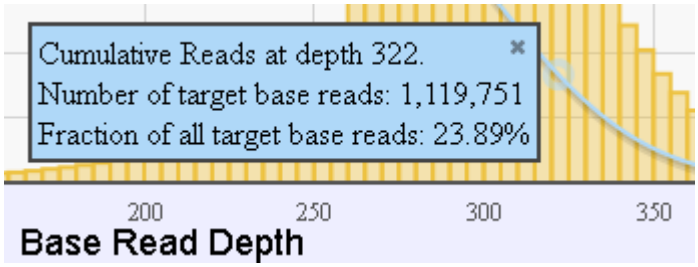


In the Depth of Coverage chart above, the left Y-axis (% reads) is the number of reads at a particular read depth (or bin of read depths) as a percentage of the total number of base reads. The right Y-axis (% cumulative reads) is the cumulative count of the number of reads at a given read depth count is at least read depth, as a percentage of the total number of reads. If your analysis includes a regions of interest file, this chart reflects only target regions (reads that fall within a region of interest).

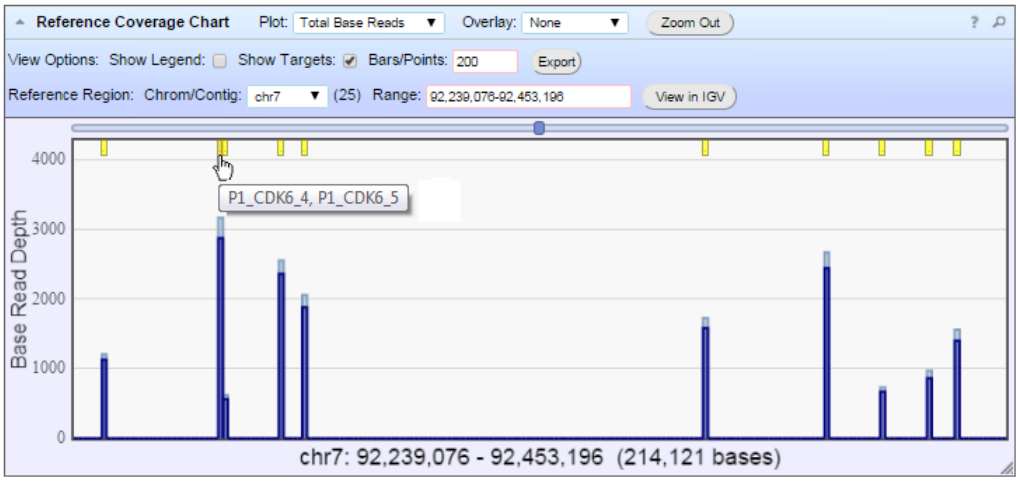
In most charts you click a data point to open a detail panel for that data:



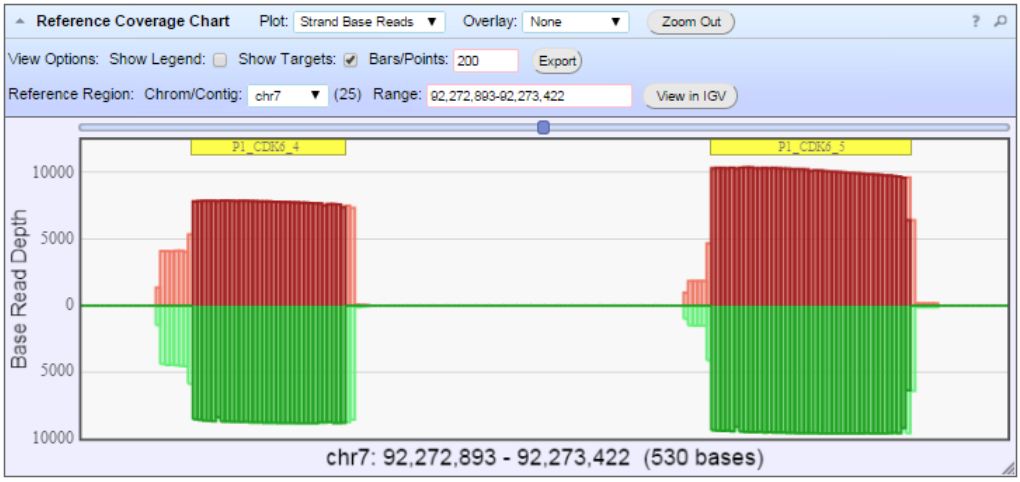
In this chart, the blue curve measures the cumulative reads at that read depth or greater. Click a point on the blue curve to open the blue detail panel for that read depth:



The following Reference Coverage Chart is shown with the **Strand Base Reads** option:



You can also zoom in to a region of interest.

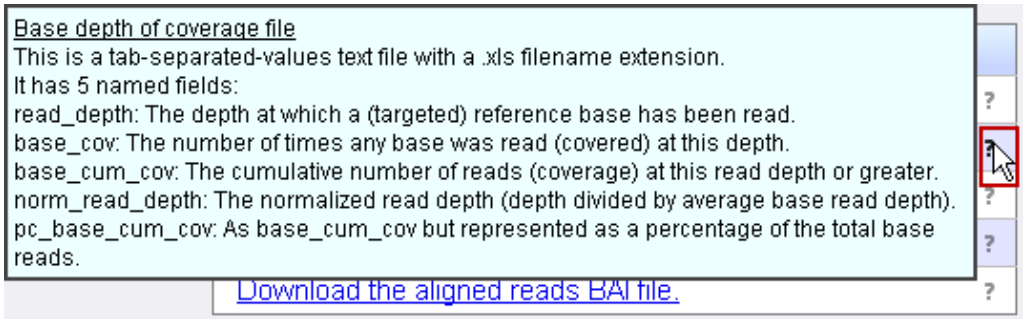


Output files

You can download plugin results files from links that are contained in the **File Links** section.

Sometimes the file name can be too long to open in applications such as Microsoft™ Excel™. To resolve this problem, right-click the file and click **Save As** to rename the downloaded file.

Click a question mark next to the file to open a description of the file:



The list of files depends on the application type selected. The following list is for an Ion AmpliSeq™ DNA run.

File	Description
Coverage statistics summary	A summary of the statistics presented in the tables at the top of the plugin report. The first line is the title. Each subsequent line is either blank or a particular statistic title followed by a colon (:) and its value.
Base depth of coverage	Coverage summary data used to create the Depth of Coverage Chart. This file contains these fields: • **read_depth** The depth at which a (targeted) reference base has been read. • **base_cov** The number of times any base was read (covered) at this depth. • **base_cum_cov** The cumulative number of reads (coverage) at this read depth or greater. • **norm_read_depth** The normalized read depth (depth divided by average base read depth). • **pc_base_cum_cov** As base_cum_cov but represented as a percentage of the total base reads.

File	Description
Amplicon coverage summary	Coverage summary data used to create the Amplicon Coverage Chart. This file contains these fields: • **contig_id** The name of the chromosome or contig of the reference for this amplicon. • **contig_srt** The start location of the amplicon target region. This coordinate is 1-based, unlike the corresponding 0-based coordinate in the original targets BED file. • **contig_end** The last base coordinate of this amplicon target region. **Note:** The length of the amplicon target is given as tlen = (contig_end - contig_srt + 1). • **region_id** The ID for this amplicon as given as the 4th column of the targets BED file. • **gene_id** The gene symbol as given as the last field of the targets BED file. • **gc_count** The number of G and C bases in the target region. %GC = 100% * gc / tlen. • **overlaps** The number of times this target was overlapped by any read by at least one base. Individual reads might overlap multiple amplicons where the amplicon regions themselves overlap. • **fwd_e2e** The number of assigned forward strand reads that read from one end of the amplicon region to the other end. • **rev_e2e** The number of assigned reverse strand reads that read from one end of the amplicon region to the other end. • **total_reads** The total number of reads assigned to this amplicon. This value equals (fwd_reads + rev_reads) and is the field that rows of this file are ordered by (then by contig id, srt and end). • **fwd_reads** The number of forward strand reads assigned to this amplicon. • **rev_reads** The number of reverse strand reads assigned to this amplicon. • **cov20x** The number of bases of the amplicon target that had at least 20 reads. • **cov100x** The number of bases of the amplicon target that had at least 100 reads. • **cov500x** The number of bases of the amplicon target that had at least 500 reads.

File	Description
Chromosome base coverage summary	Base reads per chromosome summary data used to create the default view of the Reference Coverage Chart. This file contains these fields: • **chrom** The name of the chromosome or contig of the reference. • **start** The coordinate of the first base in this chromosome. This is always 1. • **end** The coordinate of the last base of this chromosome. Also its length in bases. • **fwd_reads** The total number of forward strand base reads for the chromosome. • **rev_reads** The total number reverse strand base reads for the chromosome. • **fwd_ontrg** (if present) The total number of forward strand base reads that were in at least one target region. • **seq_reads** The total sequencing (whole) reads that are mapped to individual contigs.
Aligned reads BAM file	Contains all aligned reads used to generate this report, in BAM format. This is the same file that can be downloaded from the main report (for the specific barcode). See the current SAM tools documentation for more file format information.
Aligned reads BAI file	Binary BAM index file as required by some analysis tools and alignment viewers such as IGV. This is the same file that can be downloaded from the main report (for the specific barcode).

ampliSeqRNA plugin

The ampliSeqRNA plugin is used with the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit, Ion AmpliSeq™ Transcriptome Mouse Gene Expression Kit, or Ion AmpliSeq™ RNA panels. The plugin generates statistics, downloadable data files, and interactive visualizations that represent targeted RNA transcripts.

Use the ampliSeqRNA plugin on runs that are aligned to the human or mouse transcriptome references and appropriate target regions files, which are listed in the following table. For more information on the references and target regions files, see the *Torrent Suite™ Software 5.10 Help* (Pub. No. MAN0017597).

Reference	Target Regions File
hg19_AmpliSeq_Transcriptome_ERCC_v1	hg19_AmpliSeq_Transcriptome_21K_v1
hg19_AmpliSeq_Transcriptome_V1.1	hg19_AmpliSeq_Transcriptome_21K_v1
hg19_AmpliSeq_Transcriptome_V1.2	hg19_AmpliSeq_Transcriptome_v1.2
AmpliSeq_Mouse_Transcriptome_V1	AmpliSeq_Mouse_Transcriptome_v1

ampliSeqRNA plugin configuration

The configuration options for the ampliSeqRNA plugin are described in the following table. This plugin cannot be configured globally.

Note: You can change the **Reference Genome** used in the plugin run, for example from hg19 to mm10 if you edit the run report, then reanalyze the raw reads. Alternatively, you can use reanalyze the run. For details, see the *Torrent Suite™ Software 5.10 Help* (Pub. No. MAN0017597).

Setting	Description
The following settings can be configured when you or select the ampliSeqRNA plugin as part of a Planned Run or Planned Run template.	
Filter Barcodes	Select this checkbox to remove whole barcodes from subsequent analyses if they have a relatively low number of reads, such as those that can result from barcode contamination. A warning appears in the barcode summary report if any barcodes were discounted from the analysis. This setting is ignored for runs not employing barcodes. Typically, the Filter Barcodes option is not needed if your Planned Run specifies which samples to associate with specific barcodes.
ERCC Tracking	Select this checkbox if your Ion AmpliSeq™ RNA targets (amplicons) were spiked with ERCC tracking targets.

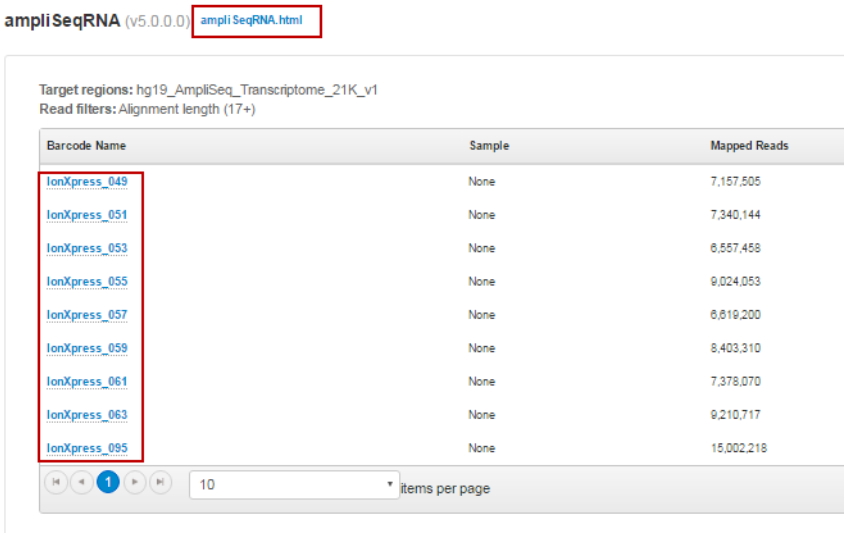
Setting	Description
The following settings can be configured when you run the ampliSeqRNA plugin manually.	
Library Type	ampliSeqRNA is selected automatically and is currently the only **Library Type** that the ampliSeqRNA plugin is designed to work with. Note: If the Planned Run specified a different application, a dialog box will warn you that the plugin may not be appropriate for the run.
Targeted Regions	This is set to the target regions file used in the Planned Run. **Note:** You can override the default Target Regions setting that each barcode uses. This might be useful to specify a subset of genes of interest, or to correct the original Planned Run.
Filter Barcodes	Select this checkbox to remove whole barcodes from subsequent analyses. Typically, the **Filter Barcodes** option is not needed if your Planned Run specifies which samples to associate with specific barcodes.
ERCC Tracking	Select this checkbox if your Ion AmpliSeq™ RNA targets (amplicons) were spiked with ERCC tracking targets.

Review ampliSeqRNA plugin results

The ampliSeqRNA plugin generates an initial summary report that lists the samples, the number of mapped reads, the percent of valid reads, and the percent of targets detected. A series of log2 reads-per-million (RPM) pair correlation plots are included for rapid correlation analysis. Microsoft™ Excel™-compatible reports are also generated, including differential expression tables. Additional details about read coverage are also provided on a per-barcode basis, along with a list of gene annotations for each sequenced region.

After the sequencing run completes, review the plugin results in the report summary.

1. In the **Data** tab, click **Completed Runs & Reports**.
2. In the list of runs, locate the run of interest, then click the link in the **Report Name** column.
3. In the left navigation menu, click **ampliSeqRNA** to view the plugin results.



- Click the **ampliSeqRNA.html** link to open the **ampliSeqRNA Report – Barcode Summary** for all barcodes.
- In the barcode table, click individual barcode names to see the results for an individual barcode.
- Click the **Distribution Plots**, **Correlation Heatmap**, **Correlation Plot**, and **Gene Heatmap** tabs to review the data graphically.

Graphical report	Description
Distribution Plots	
Reads Alignment Summary	A graphical summary of the number of mapped and unmapped reads across barcodes, as reported in the **Barcode Summary** table.
Distribution of Gene Reads	Distribution of genes across barcodes showing the frequency of numbers of genes having similar log10 read counts. All curves are plotted on the same axis scale. The counts data are fitted to a Gaussian kernel using the default R 'density' function.

Graphical report	Description
Correlation Heatmap	A heatmap of Spearman correlation r-values for comparing log2 RPM reads pair correlation barcodes, with dendrogram reflecting ordering of barcodes as being most similar by these values.
Correlation Plot	
Barcode read pair correlation plot	Lower panels show log2(RPM+1) values plotted for each pair of barcodes, with linear least squares regression line overlaid and line slope reported. Upper panels show Pearson correlation r-values for the regression line. Diagonal panels show the frequency density plot for the individual log(RPM+1) values for each barcode. (If only one barcode has reads, a density plot is displayed.) Click the plot to open an expanded view.
Gene Heatmap	
Gene Representation Heatmap	Displays 250 genes showing the most variation in representation across barcodes as measured by the coefficient of variation (CV) of normalized read counts for genes that have at least one barcode with at least 100 RPM reads, plotted using log10 of those counts. For this plot, barcodes are omitted if they have $<10^5$ total reads.

- Click the links at the bottom of the report to download associated report files.

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, see the "Documentation and Support" section in this document.

Chemical safety

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

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

Biological hazard safety



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

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

Documentation and support

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

- Worldwide contact telephone numbers
- Product support information
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- 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/or its affiliate(s) 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 any questions, please contact Life Technologies at **www.thermofisher.com/support**.