ion torrent

Oncomine™ tumor specific panels

USER GUIDE

Catalog Number A35121

Publication Number MAN0018518

Revision D.0

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

ThermoFisher SCIENTIFIC

Life Technologies Corporation | 6055 Sunol Blvd | Pleasanton, CA 94566 USA
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.

Revision history: Pub. No. MAN0018518

Revision	Date	Description
D.0	18 December 2020	Updated to include: • Oncomine™ tumor specific RNA panel • Oncomine™ Homologous Recombination Repair Pathway Predesigned panel • New Ion Reporter™ Software 5.16 analysis workflows • Instruction to create panel-specific analysis workflows for predesigned and custom Oncomine™ tumor specific panels. • Automated library preparation on the Ion Chef™ System
C.0	22 September 2020	Updated to include new Ion Reporter™ Software 5.14 analysis workflows and instruction to create panel-specific analysis workflows for custom Oncomine™ tumor specific panels.
B.0	16 January 2020	Updated for product launch to include all 10 DNA Oncomine™ tumor specific panels. • Oncomine™ Bladder • Oncomine™ BRCA Expanded • Oncomine™ Colorectal and Pancreatic • Oncomine™ Gastric and Esophageal • Oncomine™ Gynecological • Oncomine™ Kidney • Oncomine™ Liver • Oncomine™ Lymphoma • Oncomine™ Melanoma • Oncomine™ Prostate
A.0 – Early Access	14 November 2019	New user guide for early access.

Contents

CHAPTER 1 Product information 7

Product description 7
Oncomine™ tumor specific panels 8
Ion AmpliSeq™ Library Kits 9
Required materials not supplied 11
Recommended materials and equipment 12
Ion AmpliSeq™ workflow 14
Procedure overview 15

CHAPTER 2 Order a core panel 17

Order a core or predesigned Oncomine™ tumor specific panel 17

CHAPTER 3 Order a predesigned or custom panel 19

Order a custom Oncomine™ tumor specific panel 19
Oncomine™ tumor specific panel files 24
Create an Ion Reporter™ Software analysis workflow for your predesigned or custom Oncomine™ tumor specific panel 25

CHAPTER 4 Procedural guidelines 31

Procedural guidelines 31
Guidelines for RNA isolation, quantification, and input 31
Guidelines for DNA isolation, quantification, and input 32
Guidelines for library preparation 32

CHAPTER 5 Automated library preparation on the Ion Chef™ System 34

Automated RNA library preparation 34
Reverse transcribe RNA for Chef Ready library preparation 34
Ion Chef™ Instrument setup information for automated RNA library preparation 36
Automated DNA library preparation 36
Add sample DNA to an IonCode™ Barcode Adapters 96-well PCR plate 36
Ion Chef™ Instrument setup information for automated library preparation 38

CHAPTER 6 Manual library preparation 39

RNA library preparation 39
Reverse transcribe RNA for manual library preparation 39
Prepare cDNA target amplification reactions 40
Amplify the cDNA targets 41
Combine cDNA target amplification reactions 42
DNA library preparation 43
Prepare DNA target amplification reactions 43
Amplify the targets 44
Combine DNA target amplification reactions 45
Partially digest the amplicons 45
Ligate adapters to the amplicons and purify 46
Ion Xpress™ Barcode Adapters only: Combine and dilute adapters 46
Perform the ligation reaction 47
Purify the unamplified library 48
Transfer the DNA amplicons 49
Elute and dilute the library 50
Quantify the library by qPCR and calculate the dilution factor 50

CHAPTER 7 Create a Planned Run 53

About Planned Runs 53
Update Oncomine™ tumor specific panel templates in Torrent Suite™ Software 54
Download and install BED files 54
Create a Planned Run template for your Oncomine™ tumor specific panel 55
Create a Planned Run 57

CHAPTER 8 Combine and sequence libraries 62

Guidelines for combining libraries 62
Recommended maximum number of individual Oncomine™ tumor specific panels per chip 63
Combine DNA libraries 63
Combine RNA libraries 65
Combine paired DNA and RNA libraries 66
Guidelines for templating and sequencing 68

CHAPTER 9 Variant analysis 69

Analysis workflows in Ion Reporter™ Software 5.16 69
Analysis workflows in Ion Reporter™ Software 5.14 72
Custom Ion Reporter™ Software 5.14 analysis workflows for use with Oncomine™ tumor specific panels 73
Manually launch an analysis 74

View results 75
Fusion results 77
Oncomine™ BRCA analysis results 83
View Oncomine™ BRCA analysis results 83
Visualize the BRCA report 85
Generate an Analysis Results Report 90
Download Ion Reporter™ annotation VCF or TSV files 91

APPENDIX A Tips and troubleshooting 93

Tips 93
Troubleshooting 95
Library yield and quantification 95
Low amplicon uniformity (DNA only) 97
Other 99

APPENDIX B Supplemental information 101

Configure the IonReporterUploader plugin in Torrent Suite™ Software 101

APPENDIX C Quantify the amplified library with a Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ instrument 102

Amplify the library 102
Purify the amplified library 103
First-round purification 103
Second-round purification 104
Qubit™ Fluorometer: Quantify the library and calculate the dilution factor 105
Agilent™ 2100 Bioanalyzer™ instrument: Quantify the library and calculate the dilution factor 106
Store libraries 106

APPENDIX D Equalize the library 107

Before you begin 107
Amplify the library 108
Wash the Equalizer™ Beads *(if not previously performed)* 109
Add Equalizer™ Capture to the amplified library 109
(Optional) Combine captured libraries 109
Add Equalizer™ Beads and wash 110
Elute the Equalized library 111
Store libraries 111

APPENDIX E Oncomine™ tumor specific Sample ID Panel 112

Using the Ion AmpliSeq™ Sample ID Panel 112

APPENDIX F Review sequencing results 114

coverageAnalysis plugin 114
coverageAnalysis plugin configuration 114
Review coverageAnalysis plugin results 116

APPENDIX G Oncomine™ Variant Annotator plugin criteria 126

Oncomine™ tumor specific assays with Ion Reporter™ Software 5.16 126
Oncomine™ tumor specific assays with Ion Reporter™ Software 5.14 128
Oncomine™ tumor specific assays with Ion Reporter™ Software 5.12 131

APPENDIX H Safety 133

Chemical safety 134
Biological hazard safety 135

APPENDIX I Documentation and support 136

Related documentation 136
Customer and technical support 137
Limited product warranty 137

1 Product information

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

Product description

Oncomine™ tumor specific panels are small (15–30 genes), curated targeted next-generation sequencing (NGS) panels based on Ion AmpliSeq™ technology. They provide an end-to-end solution for molecular profiling and clinical research of specific tumors (bladder, prostate, gastric, and others) from formalin-fixed, paraffin-embedded (FFPE) tissue samples. Each panel is customizable from over 250 genes in inventory, and include primers from the Ion AmpliSeq™ Sample ID Panel to prevent research sample misidentification and provide gender determination. For panels associated with solid tumors, an RNA panel can also be added to detect known, novel, and rare fusions across 49 pan-cancer genes. Each Oncomine™ tumor specific panel is ordered from **AmpliSeq.com** and manufactured on demand in one of five pack sizes.

Included in your purchase with an Oncomine™ tumor specific panel is either the Ion AmpliSeq™ Library Kit Plus for manual library preparation or Ion AmpliSeq™ Kit for Chef DL8 for automated library preparation. The panels are compatible with Ion Torrent™ Dual Barcodes, IonCode™ Barcode Adapters, or Ion Xpress™ Barcode Adapters, so that multiple libraries can be combined and sequenced on a single Ion 530™ or Ion 540™ Chip to minimize the per-sample sequencing cost.

This guide covers library preparation from DNA and RNA using the following products:

- Oncomine™ tumor specific panels (DNA and RNA, Cat. Nos. various)
- Ion AmpliSeq™ Library Kit Plus (Cat. Nos. 4488990, A35907, A38875)
- Ion AmpliSeq™ Kit for Chef DL8 (Cat. No. A29024)
- Ion Torrent™ NGS Reverse Transcription Kit (Cat. No. A45003)
- Ion Torrent™ Dual Barcode Kit 1–96 (Cat. No. A39360)
- IonCode™ Barcode Adapters (Cat. Nos. various)
- Ion Xpress™ Barcode Adapters (Cat. Nos. various)
- Ion Library Equalizer™ Kit (Cat. No. 4482298)

Oncomine™ tumor specific panels

Each Oncomine™ tumor specific panel is optimized for use with FFPE-derived DNA and RNA, and can be designed and ordered at **AmpliSeq.com**. Cancer-type specific core panels can be customized by adding gene targets from inventory or removing genes. Each panel consists of two DNA primer pools. The number of primer pairs per pool depends on the number of genes in the design. Oncomine™ tumor specific panels are for Research Use Only. Not for use in diagnostic procedures.

Panels can be ordered in two formats and multiple reaction numbers.

Format	Number of reactions	Method
Manual library preparation	24, 96, or 384 reactions	Ion AmpliSeq™ Library Kit Plus
Automated library preparation using the Ion Chef™ System	32, or 128 reactions	Ion AmpliSeq™ Kit for Chef DL8[1]

[1] See *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432).

Available Oncomine™ tumor specific panel configurations

Panel	Number of amplicons[1]	Storage[2]
Oncomine™ tumor specific core panels		
DNA panels		
Oncomine™ Bladder	887	–30°C to –10°C
Oncomine™ BRCA Expanded	1011	
Oncomine™ Colorectal and Pancreatic	898	
Oncomine™ Gastric and Esophageal	920	
Oncomine™ Gynecological	846	
Oncomine™ Kidney	851	
Oncomine™ Liver	956	
Oncomine™ Lymphoma	957	
Oncomine™ Melanoma	909	
Oncomine™ Prostate	937	
RNA panels		
Oncomine™ tumor specific RNA panel	N/A	–30°C to –10°C

Available Oncomine tumor specific panel configurations *(continued)*

Panel	Number of amplicons[1]	Storage[2]
Oncomine™ tumor specific predesigned panels		
DNA panels		
Oncomine™ Homologous Recombination Repair Pathway Predesigned	1481	–30°C to –10°C

[1] The number of amplicons listed includes the sample ID panel amplicons.
[2] Shipped at ambient temperature. Store as indicated.

Ion AmpliSeq™ Library Kits

Ion AmpliSeq™ library kits are included with purchase of an Oncomine™ tumor specific assay. The library kit included is dependent upon the preparation method (manual or on an Ion Chef™ System) and reaction size, and is selected during the ordering process. For more information on the ordering process, see Chapter 2, “Order a core panel” or Chapter 3, “Order a predesigned or custom panel”.

Ion AmpliSeq™ Library Kit Plus

Ion AmpliSeq™ Library Kit Plus 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. We recommend preparing libraries from 3,000 copies (10 ng) DNA or cDNA.

- The Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990) provides reagents for manually preparing 24 libraries for 1- or 2-pool panels.
- The Ion AmpliSeq™ Library Kit Plus (Cat. No. A35907) provides reagents for manually preparing 96 libraries for 1- or 2-pool panels.
- The Ion AmpliSeq™ Library Kit Plus (Cat. No. A38875) provides reagents for manually preparing 384 libraries for 1- or 2-pool panels.

Component	Amount			Storage
	Cat. No. 4488990 (24 reactions)	Cat. No. A35907 (96 reactions)	Cat. No. A38875 (384 reactions)	
5X Ion AmpliSeq™ HiFi Mix (red cap)	120 µL	480 µL	4 × 480 µL	–30°C to –10°C
FuPa Reagent (brown cap)	48 µL	192 µL	4 × 192 µL	
Switch Solution (yellow cap)	96 µL	384 µL	4 × 384 µL	
DNA Ligase (blue cap)	48 µL	192 µL	4 × 192 µL	
25X Library Amp Primers (pink cap)	48 µL	192 µL	4 × 192 µL	
1X Library Amp Mix (black cap)	1.2 mL	4 × 1.2 mL	16 × 1.2 mL	
Low TE	6 mL	2 × 6 mL	8 × 6 mL	15°C to 30°C[1]

[1] Can be stored at –30°C to –10°C.

Ion AmpliSeq™ Kit for Chef DL8 (automated library preparation)

IMPORTANT! Upon arrival, inspect all consumables and contact Technical Support if any of the components have been damaged during shipping. Store all consumables and cartridges under the recommended conditions and in an upright position. Ion AmpliSeq™ Chef Solutions DL8 cartridges are shipped at ambient temperature, but need to be stored at 2°C to 8°C upon arrival.

The Ion AmpliSeq™ Kit for Chef DL8 (Cat. No. A29024) includes the reagents and materials for automated preparation of up to 8 barcoded Ion AmpliSeq™ libraries per Ion Chef™ run. The kit provides reagents and materials sufficient for 4 runs, giving the user the ability to prepare up to 32 barcoded libraries. Fewer than 8 samples can be processed in a run, but a run consumes kit reagents for 8 samples regardless of sample number.

Component	Amount		Storage
	32 rxns	128 rxns	
Ion AmpliSeq™ Chef Reagents DL8	4 cartridges	16 cartridges	–30°C to –10°C
Ion AmpliSeq™ Chef Solutions DL8	4 cartridges	16 cartridges	2°C to 8°C[1]
Ion AmpliSeq™ Chef Supplies DL8 (per insert) • Ion AmpliSeq™ Tip Cartridge L8 • PCR Frame Seal [2] • Enrichment Cartridge	1 box with 4 inserts	4 boxes with 4 inserts	15°C to 30°C
IonCode™ 0101–0132 in 96 Well PCR Plates (dried) Set includes 4 PCR plates: • IonCode™ 0101–0108 in 96 Well PCR Plate (red) • IonCode™ 0109–0116 in 96 Well PCR Plate (yellow) • IonCode™ 0117–0124 in 96 Well PCR Plate (green) • IonCode™ 0125–0132 in 96 Well PCR Plate (blue)	1 set of 4 plates	4 sets of 4 plates	15°C to 30°C

[1] Ion AmpliSeq™ Chef Solutions DL8 cartridges are shipped at ambient temperature, but need to be stored at 2°C to 8°C upon arrival.
[2] Use scissors to open the plastic bag containing the PCR Frame Seal . Tearing the bag open may damage the seal.

Required materials not supplied

In addition to a panel and library kit, 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
Ion Library TaqMan™ Quantitation Kit[2]	4468802
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**)
Pipettors, 2–200 µL, and low-retention filtered pipette tips	(**fisherscientific.com**)
(RNA only) Ion Torrent™ NGS Reverse Transcription Kit	A45003
Barcode adapters	
IonCode™ Barcode Adapters 1–384 Kit	A29751
Ion Xpress™ Barcode Adapters Kit	Various
Ion Torrent™ Dual Barcode Kit 1–96	A39360

[1] Supported but no longer available for purchase.

[2] Requires a real-time PCR instrument, see “Recommended materials and equipment”.

Recommended materials and equipment

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

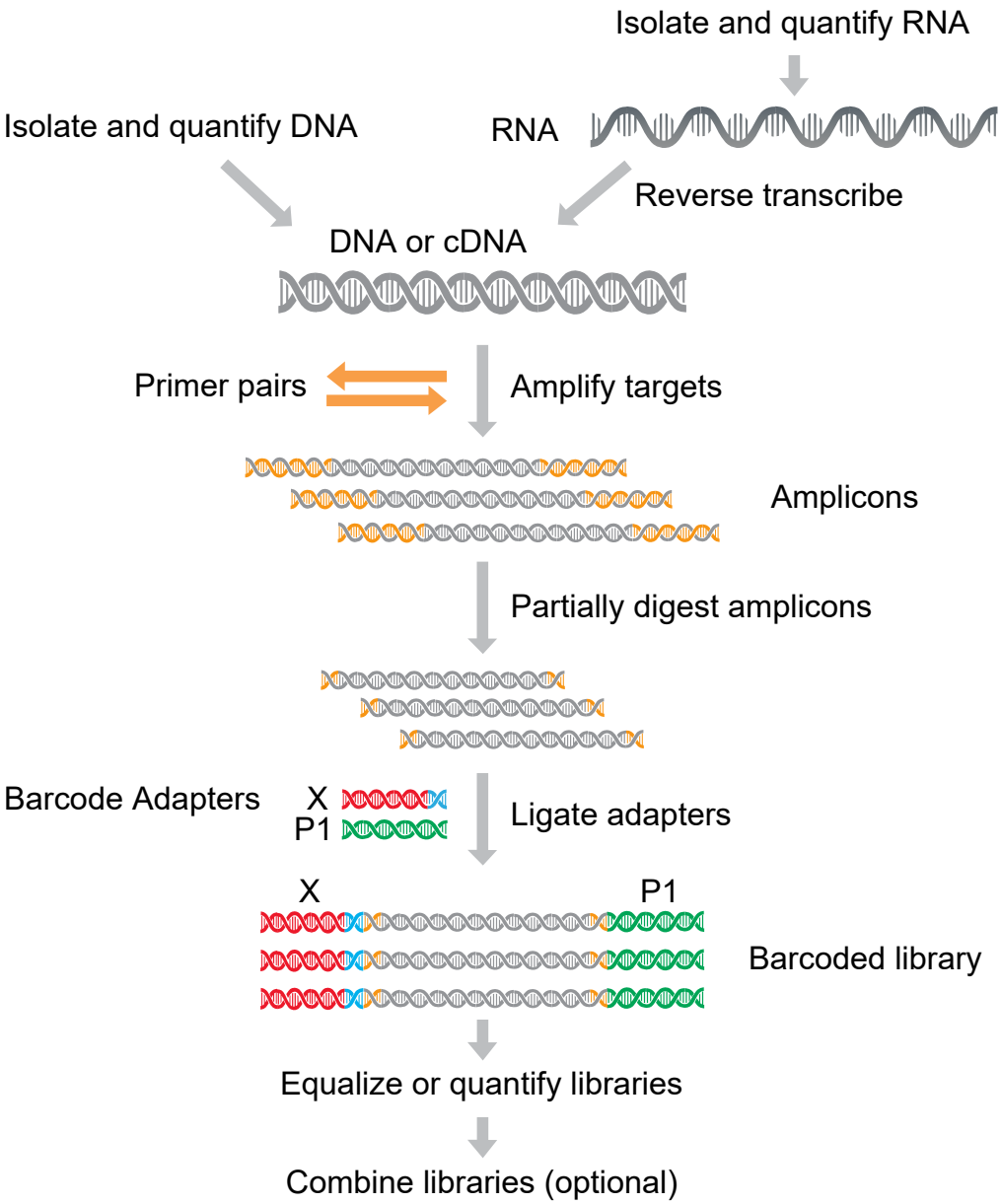
Item	Source
Additional equipment	
One of the following Applied Biosystems™ real-time PCR instruments: • 7500 Real-Time PCR System • 7900HT Fast Real-Time PCR System[1] • StepOne™ Real-Time PCR System • StepOnePlus™ Real-Time PCR System • ViiA™ 7 Real-Time PCR System • QuantStudio™ 3 Real-Time PCR System • QuantStudio™ 5 Real-Time PCR System • QuantStudio™ 7 Flex Real-Time PCR System • QuantStudio™ 12K Flex Real–Time PCR System	Various
Fisher Scientific™ Mini Plate Spinner Centrifuge, or equivalent 96-well plate centrifuge	14-100-143 (**fisherscientific.com**)
Qubit™ 4 Fluorometer[2]	Q33238
Agilent™ 2100 Bioanalyzer™ and appropriate assay kit	G2939BA (**agilent.com**)
MicroAmp™ Adhesive Film Applicator	4333183
Nucleic acid isolation	
RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	AM1975
RecoverAll™ Multi-Sample RNA/DNA Workflow	A26069
MagMAX™ FFPE Total Nucleic Acid Isolation Kit	4463365
MagMAX™ FFPE DNA/RNA Ultra Kit	A31881
PureLink™ Genomic DNA Mini Kit	K1820-00
Nucleic acid quantification	
TaqMan™ RNase P Detection Reagents Kit *(Recommended for DNA only)*	4316831
Qubit™ dsDNA HS Assay Kit	Q32851 Q32854
Qubit™ RNA HS Assay Kit	Q32852 Q32855

(continued)

Item	Source
Library quantification	
Agilent™ High Sensitivity DNA Kit	5067-4626 (**agilent.com**)
Ion Library Equalizer™ Kit	4482298
Controls	
Control DNA (from CEPH Individual 1347-02)	403062
AcroMetrix™ Oncology Hotspot Control	969056
Seraseq™ Fusion RNA Mix v4	0710-0497 **seracare.com**
Horizon™ ALK RET ROS RNA fusion (EML4-ALK, CCDC6-RET, and SLC34A2-ROS1)	HD784 **horizondiscovery.com**

[1] Supported but no longer available for purchase.
[2] Qubit™ 2.0 & Qubit™ 3.0 Fluorometers are supported but no longer available for purchase.

Ion AmpliSeq™ workflow



Procedure overview

Manual library preparation

Order a predesigned, core or customized tissue specific panel.

a. Download tumor specific panel files.
b. *(Customized and predefined panels only)* Download BAM files for creation of tumor specific panel copy number baseline.
c. *(Customized and predefined panels only)* Create panel-specific Ion Reporter™ Software analysis workflow.
d. Import panel-specific BED file into Torrent Suite™ Software and create panel-specific planned run.

Isolate sample DNA or RNA.

If starting with RNA, reverse-transcribe to make cDNA.

Prepare panel specific libraries

a. Amplify target regions from DNA or cDNA with the Ion AmpliSeq™ Library Kit Plus.
b. Partially digest amplicons with FuPa Reagent.
c. Ligate adapters with Switch Solution and DNA Ligase, then purify.

Quantify or normalize the libraries.

- Without further amplification, quantify libraries by qPCR and dilute to 50 pM.
- Normalize libraries to ~100 pM without the need for quantification or dilution using the Ion Library Equalizer™ Kit.
- Quantify libraries using the Qubit™ Fluorometer or the Agilent™ 2100 Bioanalyzer™ instrument and dilute to 50 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.

Create a planned run

Planned Runs are digital instructions that are created in Torrent Suite™ Software for controlling the template preparation and sequencing instruments.

(continued)

Manual library preparation

Combine and sequence libraries

a. Combine barcoded libraries
b. Templating
c. Sequencing
d. Data analysis

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

2 Order a core panel

Order a core or predesigned Oncomine™ tumor specific panel

Oncomine™ tumor specific panels are available at **AmpliSeq.com**. Core Oncomine™ tumor specific panels do not require modifications to the provided Torrent Suite™ Software planned run template and panel specific Ion Reporter™ Software analysis workflows. To create, order, and analyze data from a customized Oncomine™ tumor specific panel see "Order a custom Oncomine™ tumor specific panel" on page 19.

1. Sign in to your Ion AmpliSeq™ Designer account.
2. In the **Home** screen, in the **Oncomine™ tumor specific panels** pane, click **Browse tumor types**.
3. In the **Oncomine™ tumor specific panels for cancer research** screen, click within the pane of a **Core Panel** or a **Predesigned Panel** to select the panel of interest, then click **Next**.

   The panel design screen opens, where you can view both DNA and RNA panel details, download panel-specific files, and customize the panel.

   Note: The Oncomine™ tumor specific RNA panel is not available with the Oncomine™ Lymphoma panel.

   ---

4. Download panel-specific files for the locked 🔒 design.
   a. Click **Download Files**, then in the **Download Files** dialog select either **DNA only** or **DNA and Fusions** depending on the panels chosen.
   b. Click **Download**.

   A ZIP file that contains files required for creating DNA only or DNA and Fusions Torrent Suite™ Software run plans is downloaded to your local storage.
5. When you are ready to order the panel, click **Preview Order**.
6. In the **Order options: panel type** dialog, select **DNA only** or **DNA and Fusions**, then click **Next**.
7. In the **Order options: GeneStudio** dialog, click **Next**.
8. In the **Library preparation format/reactions: GeneStudio** dialog, select **Manual** or **Ion Chef™** format, then select the number of reactions. Click **Next**.

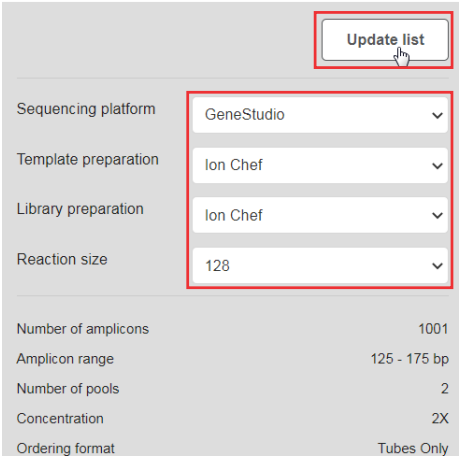
9. In the **Order summary** dialog, review order details, then proceed to finalize your order in one of the following ways.
   - To view and select additional consumables or change ordering options, select **List recommended consumables**, then click **Preview**.
   - To proceed directly to cart, click **Proceed to cart** (**Request a quote** in some regions). You are redirected to the **Cart** on thermofisher.com, where you can add or remove items from your order as needed, view product price estimates, and begin the checkout process.

   Note: If you proceed directly to cart, you will not be able to change sequencing platform, template preparation, or ordering format selections for your design.

10. In the **Order preview** screen, review design order details, then if needed, edit ordering options and select additional consumables to add to your order.
    a. In the ordering options pane, click **Edit list**, then make any desired changes for the **Sequencing platform**, **Template preparation**, and **Ordering format**. When finished, click **Update list**.

    b. In the **Library preparation**, **Template preparation and sequencing**, **Barcodes**, and **Chips** panes, in the row of each kit that you want to add to your order, enter the quantity in the **Qty** field.

    c. When finished, click **Add all to cart**.



You are redirected to the **Cart** on thermofisher.com, where you can add or remove items from your order as needed, view product price estimates, and begin the checkout process.

Note: Removal of the selected library kit may result in an invalid order and the order will not be processed. Any barcode kit, templating kits and chips can be removed without affecting the ordering process.

The core Oncomine™ tumor specific panels are ready to use with the provided Torrent Suite™ Software planned run templates and panel specific Ion Reporter™ Software analysis workflows. Proceed to Chapter 7, “Create a Planned Run” to continue with templating and sequencing.

Predesigned panels do not have dedicated Ion Reporter™ Software analysis workflows. Analysis is supported with the BED file provided, similar to a custom panel. For more informationon creating an Ion Reporter™ Software analysis workflow, see “Create an Ion Reporter™ Software analysis workflow for your predesigned or custom Oncomine™ tumor specific panel” on page 25.

3 Order a predesigned or custom panel

Order a custom Oncomine™ tumor specific panel

You can customize Oncomine™ tumor specific panels by adding or removing genes. You can also customize previously created and locked custom panels. The core and predesigned Oncomine™ tumor specific panels are locked and ready to order. To customize a locked panel, you must first unlock the design. In an unlocked state, you can add or remove genes from the panel and edit the design name and details. When you finalize your customized panel design, you must lock the design in order to download panel-specific files, view amplicon distribution, and proceed with the purchase of your panel.

Note: The Oncomine™ tumor specific RNA panel can not be customized and is not available with the Oncomine™ Lymphoma panel or with custom panels created from scratch using a gene list.

If you want to customize a previously created and *locked* custom design, click **My Designs** ▸ **Oncomine Tumor Specific**, then in the **Design** column, click the name of the design of interest. In the panel design screen, click **More Actions** ▸ **Copy Design**, then proceed to substep 4a.

1. Sign in to your Ion AmpliSeq™ Designer account.
2. In the **Home** screen, in the **Oncomine™ tumor specific panels** pane, click **Browse tumor types**.
3. In the **Oncomine™ tumor specific panels for cancer research** screen, click within the pane of a **Core Panel** or a **Predesigned Panel** to select the panel of interest, then click **Next**.

   Alternatively, if interested in only a DNA panel, click **Or add gene list** to create a custom panel by inputting or uploading a gene list.

   The panel design screen opens, where you can view both DNA and RNA panel details, download panel-specific files, and customize the panel design.

   Note: The Oncomine™ tumor specific RNA panel is not available for order with panels created from a user provided gene list.

   ---

4. In the panel design screen, click **Customize**, then in the **Customize** window, enter a panel name and details.
   a. *(Required)* In the **Name** field, enter a unique name for your custom panel.

      The panel name can be up to 20 characters in length and must be unique.

   b. *(Optional)* In the **Details** field, enter additional information for your custom panel.
5. Click **Save**.

   The selected panel design is unlocked and enabled for customization. Unlocked designs display 🔓 next to the panel name in the panel design screen.

6. Add or remove genes of interest.

Option	Description
Add core genes or associated research genes	In the **Core Genes** section or in the **Associated Research Genes** section, use one of the following approaches to add one or more genes to your new customized panel. • Select a checkbox for each gene that you want to include in your panel (see callout 3 in Figure 1). • Click **Include All** to select all of the displayed genes to be included in your panel. Genes that are included in a panel are marked in green (see callout 1 in Figure 1).
Add in-stock genes	1. Click **Add In Stock Genes** (see callout 6 in Figure 1). The **Add In Stock Genes** window opens. 2. Manually add individual genes to your panel or input a list of genes of interest by file. • In the **Add Genes** tab, enter a gene symbol for each gene that you want to add (for example, enter AKT1). If adding more than one gene, separate each gene symbol by a comma, a space, or a new line (by pressing **Return** after each gene symbol). • In the **Upload File** tab, click **Choose File**, navigate to a CSV file that contains a list of gene symbols for genes that you want to add to your custom panel, then click **Open**. To download a template CSV file that you can copy/edit with a list of gene symbols of interest, click **Download this example**. In-stock genes are added to the **Added Genes** section (see callout 5 in Figure 1).
Remove genes	In the **Core Genes**, **Associated Research Genes**, or **Added Genes** section, use one of the following approaches to remove one or more genes from your custom panel. • Deselect a checkbox for each gene that you want to exclude from your custom panel (see callout 3 in Figure 1). • Click ✕ to remove a gene from a panel (see callout 4 in Figure 1). This action also removes a gene from the panel design screen. If you remove a gene by mistake, you can add the gene back to your custom panel using the **Add In Stock Genes** functionality. • Click **Exclude All** to exclude all core genes or associated research genes from your custom panel. Genes that are excluded from a panel appear greyed out (see callout 2 in Figure 1).

Note: The Oncomine™ tumor specific RNA panel can not be changed.

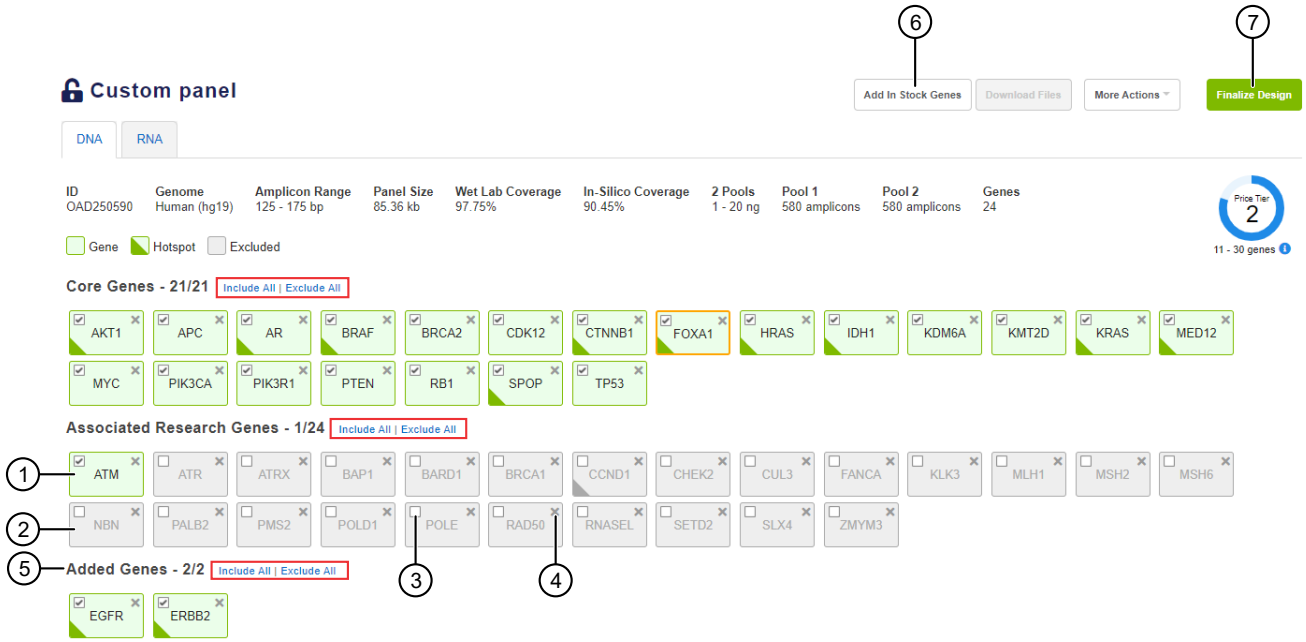


Figure 1 Custom panel design screen

(1) A gene that is included in the panel.
(2) A gene that is excluded from the panel.
(3) Select a checkbox to include a gene in the panel or deselect a checkbox to exclude a gene from the panel.
(4) Click ✕ to remove a gene from a panel and the panel design screen.
(5) **Added Genes** section displays in-stock genes that have been added to the panel.
(6) Click **Add In Stock Genes** to add one or more in-stock genes to the panel.
(7) Click **Finalize Design** to finalize and lock the design.

7. Click **Finalize Design** to lock the design, then click **Finalize Design** in the confirmation dialog (see callout 7 in Figure 1).

   It may take up to 30 minutes to finalize the design. When complete, the **Finalize Design** button is replaced by **Preview Order**.

8. Click **Download Files**. In the **Download Files** dialog select either **DNA only** or **DNA and Fusions** depending on the panels chosen, then click **Download**.

   Note: You can only download files for finalized (🔒 locked) designs.

   A ZIP file that contains files required for creating DNA only or DNA and Fusions custom run plans and Ion Reporter™ Software analysis workflows is downloaded to your local storage.

9. *(Optional)* Click **More Actions**, then select one of the following options from the dropdown list.

Option	Description
Edit Design Name & Details	*(Unlocked custom designs only)* Open the **Edit Custom Design** window and edit design name and details.
Export Targets	Export the CSV file (`<panel ID>_Oncomine_selected_targets.csv`) that contains the list of targets included in your design to your local storage.
Amplicon Distribution	View the amplicon size histogram for your design. To export the PNG file (`<panel ID>_Oncomine_amplicon_size_histogram.png`) that contains the amplicon size histogram for your design to your local storage, click **Download** in the **Amplicon Distribution** window. **Note:** The `<panel ID>_Oncomine_amplicon_size_histogram.png` is also downloaded as part of the ZIP file described in step 8.
Copy Design	*(Custom designs only)* Copy a design. The design is added to the **My Designs** list in the **Oncomine™ Tumor Specific** tab.
Sharing	*(Locked custom designs only)* Share a design. For more information, see the Ion AmpliSeq™ Designer help.
Restore	*(Unlocked custom designs only)* Restore a custom panel back to the core design.
Delete	*(Custom designs only)* Permanently delete a design.

10. After you review and finalize your design, click **Preview Order**.

11. In the **Order options: panel type** dialog, select **DNA only** or **DNA and Fusions**, then click **Next**.

12. In the **Order options: GeneStudio** dialog, click **Next**.

13. In the **Library preparation format/reactions** dialog, select **Manual** or **Ion Chef™** format, then select the number of reactions. Click **Next**.

14. In the **Order summary** dialog, review order details, then proceed to finalize your order in one of the following ways.

    - To view and select additional consumables or change ordering options, select **List recommended consumables**, then click **Preview**.
    - To proceed directly to cart, click **Proceed to cart**.

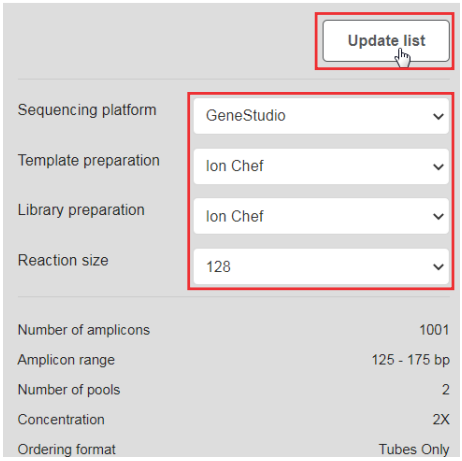
    Note: If you proceed directly to cart, you will not be able to change sequencing platform, template preparation, or ordering format selections for your design.

15. In the **Order preview** screen for the design, review order details, then if needed, edit ordering options and select additional consumables to add to your order.

    a. In the ordering options pane, click **Edit list**, then make any desired changes for the **Sequencing platform**, **Template preparation**, and **Ordering format**. When finished, click **Update list**.

    b. In the **Library preparation**, **Template preparation and sequencing**, **Barcodes**, and **Chips** panes, in the row of each kit that you want to add to your order, enter the quantity in the **Qty** field.

    c. When finished, click **Add all to cart**.



You are redirected to the **Cart** on **thermofisher.com**, where you can add or remove items from your order as needed, view product price estimates, and begin the checkout process.

Note: Removal of the selected library kit may result in an invalid order and the order will not be processed. Any barcode kit, templating kits and chips can be removed without affecting the ordering process.

For custom designs, you must download the `<panel ID>_Oncomine_Designed.bed` file for use in creating a custom Torrent Suite™ Software planned run template and Ion Reporter™ Software analysis workflow that are required for templating, sequencing, and data analysis of each customized tumor specific panel.

Oncomine™ tumor specific panel files

The following files are available for download for finalized (locked) Oncomine™ tumor specific panel designs.

File	Description
`<panel ID>_Oncomine_amplicon_insert_size_histogram.png`	A PNG image of a histogram that represents the distribution of amplicon insert sizes, excluding undigested forward and reverse primer sequences and barcode adapters.
`<panel ID>_Oncomine_amplicon_size_histogram.png`	A PNG image of a histogram that represents the distribution of amplicon sizes, including undigested forward and reverse primer sequences and barcode adapters.
`<panel ID>_Oncomine_coverage_details.csv`	A CSV file that contains exon-level coverage details, including target gene, number of amplicons that are required to cover the exon, and number of bases covered by the amplicons.
`<panel ID>_Oncomine_coverage_summary.csv`	A CSV file that contains gene-level coverage summary, including the total number of amplicons required to cover the CDS region of the gene and total number of bases covered by the amplicons.
`<panel ID>_Oncomine_Designed.bed`	A BED file that defines genomic coordinates that are targeted by the primers.
`<panel ID>_Oncomine_hotspot.bed`	A BED file that contains hotspot genomic coordinates that are covered by the `<panel ID>_Oncomine_Designed.bed` file.
`<panel ID>_Oncomine_Missed.bed`	A BED file that contains coordinates that are missed by the designer; the difference between submitted and designed.
`<panel ID>_Oncomine_Submitted.bed`	A BED file that contains genomic coordinates the primers are designed to target.
`plan.json`	A JSON file that contains panel information.

Create an Ion Reporter™ Software analysis workflow for your predesigned or custom Oncomine™ tumor specific panel

Note:

- To create an analysis workflow for any predesigned or custom Oncomine™ tumor specific panel you must first create a copy number baseline specific to that panel.
- We recommend use of the sample BAM files provided at: **apps.thermofisher.com/apps/spa/#/publiclib/datasets**, for creating your custom copy number baseline. Alternatively, you can use sample BAM files that have been run using your custom Oncomine™ tumor specific panel.

Download sample files for custom Ion Reporter™ Software analysis workflow creation

Customized Oncomine™ tumor specific panels require the user to create new analysis workflows in Ion Reporter™ Software with associated panel-specific copy number baselines for each custom panel-specific design. To create the panel-specific copy number baseline, sample BAM files and an associated CSV file containing sample attribute information are provided for download from Thermo Fisher™ Connect. Following download, the BAM and CSV files are imported into Ion Reporter™ Software for use in creating panel-specific copy number baselines.

Note:

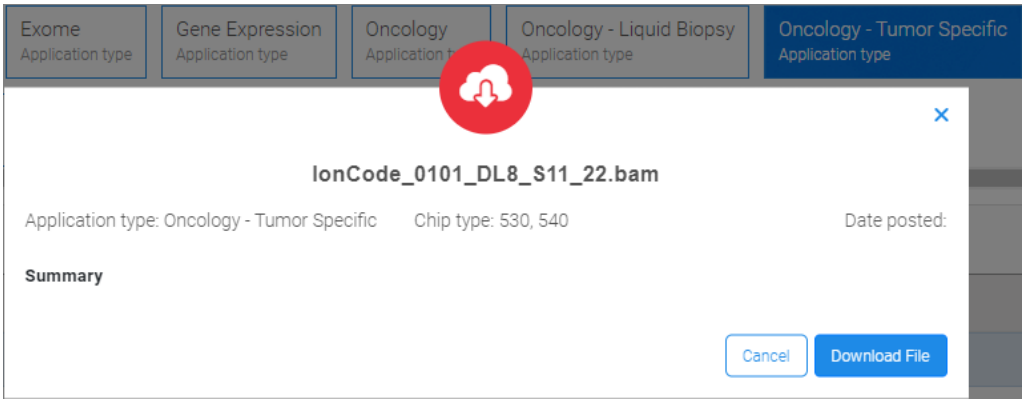
- Core Oncomine™ tumor specific panels do not require modification to the provided panel specific Ion Reporter™ Software analysis workflows.
- The total size of the sample files is more than 190 Gb. Depending on your network bandwidth, the time that it takes to download the files will vary.
- Once imported into Ion Reporter™ Software the sample BAM files are available for creation of copy number baselines for any custom tumor-specific panel design.

1. Browse to **apps.thermofisher.com/apps/spa/#/publiclib/datasets**.

2. In the **Categories** section, click **Oncology - Tumor Specific** to filter the files.
   Only the 48 sample BAM files, and `Oncomine_Tumor_Specific_Datasets.zip` are displayed. The ZIP folder contains a README file, and a template CSV file for use uploading the files to Ion Reporter™ Software.

   Note: BAM files are large, the download process may take several hours.

3. Click in the row of the file to be downloaded, then click **Download** or click (Download) directly.



Note the filepath of the directory to which the files are downloaded as this information will be needed by the Ion Reporter™ Software command-line utility for uploading the files.

Upload sample files to Ion Reporter Software

You can manually upload up to 48 BAM files into Ion Reporter™ Software to include in a panel-specific CNV baseline preset. To upload the files, use the IonReporterUploader command-line utility, which is recommended for large sample files and batch uploads. The IonReporterUploader command-line utility can be downloaded from in the Ion Reporter™ Software. For more information about the IonReporterUploader command-line utility, see the *IonReporterUploader Command-Line Utility User Guide* (Pub. No. MAN0017648), or contact your local Field Bioinformatics Specialist (FBS).

Note: The name of a BAM file that is uploaded to Ion Reporter™ Software cannot exceed 200 characters.

1. Click **Settings ( )** ▸ **Download Ion Reporter Uploader**.
2. Click the file name `IonReporterUploader_<version>-cli.zip`, then download the file to the target computer.
3. Extract the downloaded `IonReporterUploader-cli.zip` file to a convenient location on the target computer.
   If you have a 64-bit version of JAVA already installed the utility is ready to run. You can run the IonReporterUploader command-line utility, `irucli`, from the `IonReporterUploader-cli` bin directory with the `irucli.bat` (Microsoft™Windows™ operating system) or `irucli.sh` script (Linux™ operating system).
4. Create a configuration properties file.
   The configuration properties file contains the information that the IonReporterUploader command-line utility requires to connect to the Ion Reporter™ Software.

```
protocol=https
serverAddress=Your local server IP
port=443
IRVersion=5.12
userName=me@domain.com
```

5. Create a samples CSV file. This defines the samples from the BAM files that are uploaded to Ion Reporter™ Software.
   a. Download the **OTS_IRU_template.csv** file. For more information, see “Download sample files for custom Ion Reporter™ Software analysis workflow creation” on page 25.
   b. Enter the sample information.
      - First field—the sample name.
      - Second field—the filepath (on your local computer) to the data file for this sample.
      - Third field—the sample gender. Allowed values are: Male, Female or Unknown.
   c. Save the samples CSV file to your computer.
6. Open a command prompt window, then enter the following command to upload your samples.
   - `./irucli.sh -c <configuration properties file> -s <samples CSV file>` (Linux™ operating system)
   - `\path\to\irucli.bat -c <configuration properties file> -s <samples CSV file>` (Microsoft™Windows™ operating system)

For more command options, for example how to add metadata to all samples or define a log file directory, see the *IonReporterUploader Command-Line Utility User Guide* (Pub. No. MAN0017648).

Create a panel-specific CNV baseline

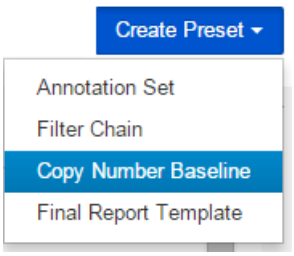
You can create a copy number baseline workflow preset in Ion Reporter™ Software to determine copy number changes in the sample of interest, without the use of a matched control. You can later add the custom copy number baseline to an analysis workflow that you can use as a baseline control for analyses.

You must use a minimum of 48 samples (the majority of which should be FFPE samples), and at least 6 of the samples must be "Normal" (without known copy number variants), to create a copy number baseline workflow preset. We recommend that you use the sample files provided for download from Thermo Fisher™ Connect. Alternatively, you can use sample BAM files that have been run using your custom Oncomine™ tumor specific panel.

If you import the custom copy number baseline, the target regions file that was used to create the copy number baseline must be available in the software to ensure that the imported copy number baseline appears in the list of available copy number baselines.

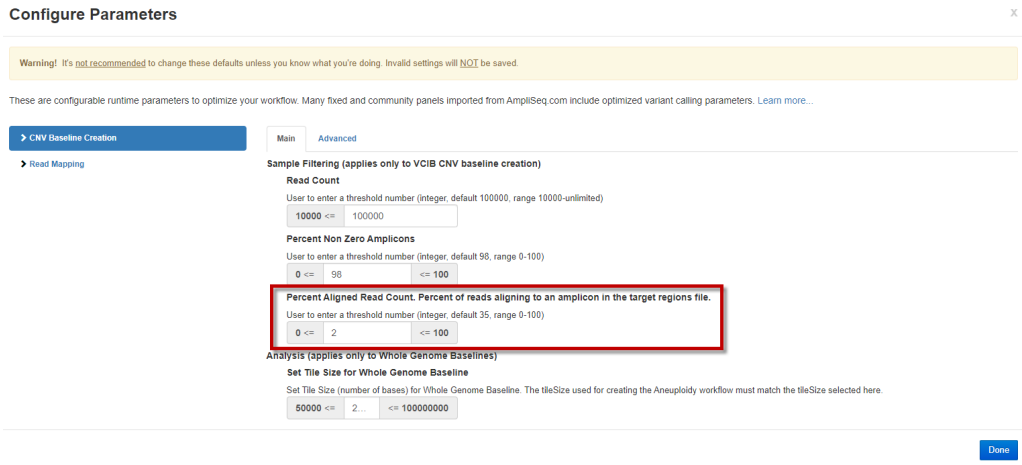
Ion Reporter™ Software provides a wizard to guide you through Copy Number Variation (CNV) Baseline creation.

1. In the **Workflows** tab, in the **Presets** screen, click **Create Preset**, then select **Copy Number Baseline** from the dropdown list.



2. Select Baseline Type: **AmpliSeq**, then select your panel-specific Target Regions file or click **AmpliSeq Import** to access your panel-specific file. Click **Next**.
3. Ensure **CNV VCIB 4.0.0.1** is selected, then click **Configure parameters**.

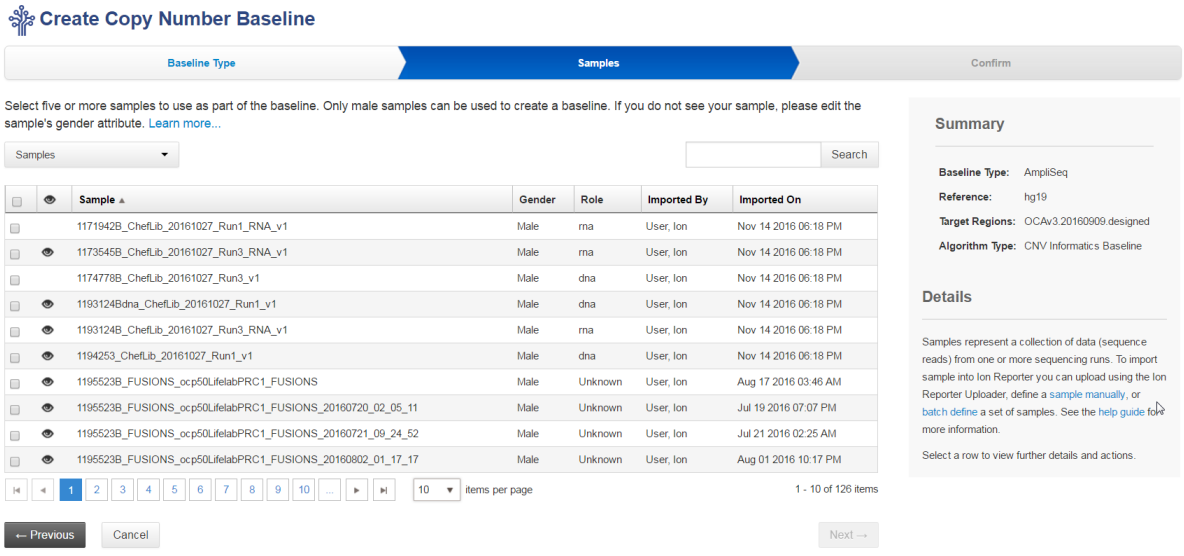
4. Enter the Percent Aligned Read Count parameter threshold number of 2, then click **Done** ▸ **Next**.



5. Select the 48 Oncomine™ tumor specific samples downloaded from Thermo Fisher™ Connect, flag any 6 of the selected samples as "Normal" by selecting the checkbox in the **Normal** column, then click **Next**.

Note:

- Male or Female gender must be specified for Normal samples, but samples that are not flagged as normal can be male, female, or unknown. You can use the Summary pane to see your totals.
- If you prefer, you can substitute up to 48 samples that you have run using your custom Oncomine™ tumor specific panel, six of which must be designated as "Normal".



6. Enter a name for your baseline, then click **Create Baseline**.

   Calculation of the new panel-specific copy number baseline can take several hours to complete. Once complete the new baseline is available to add to an analysis workflow.

Create a panel-specific Ion Reporter™ Software analysis workflow

Analysis workflows are provided in Ion Reporter™ Software 5.16 for all core Oncomine™ tumor specific panels for both the Ion 530™ Chip and Ion 540™ Chip. To create an analysis workflow for a predesigned or customized Oncomine™ tumor specific panel in Ion Reporter™ Software 5.16 or later, we recommend copy editing the Ion Reporter™ Software analysis workflow of the parent Oncomine™ tumor specific panel.

IMPORTANT! In Ion Reporter™ Software 5.14 we recommend that you use the provided Oncomine™ BRCA Expanded and Oncomine™ Lymphoma analysis workflows for analyzing core or customized Oncomine™ BRCA Expanded and Oncomine™ Lymphoma tumor specific panels rather than create a custom analysis workflow.

---

1. In the **Workflows** tab, click **Overview**.
2. In the **Workflows** table, select the Oncomine™ tumor specific analysis workflow template that you want to copy, then click **Actions** ▸ **Copy**.
   - In Ion Reporter™ Software 5.16 copy/edit the analysis workflow of the parent Oncomine™ tumor specific panel.
   - For any custom panel containing both the BRCA1 and BRCA2 genes, copy/edit the parent core panel analysis workflow if the parent core panel includes the BRCA1 and BRCA2 genes. This includes the Oncomine™ Homologous Recombination Repair Pathway Predesigned panel. If the custom panel does not include both BRCA1 and BRCA2 genes copy/edit a closely related core panel analysis workflow (Oncomine™ Prostate for example).

   The **Edit** workflow bar opens to the **Research Application** step with the **Research Application** and **Sample Group** preselected.

   Note: When you copy analysis workflow templates, you cannot change these settings.

   ---

3. Click **Next**, then in the **Reference** step, select or upload the appropriate files.

Option	Description
Select a file that has been previously uploaded to Ion Reporter™ Software.	Select the **Target Regions** and, optionally, **Hotspot Regions** files from the dropdown lists.
Upload the entire package of the Oncomine™ tumor specific panel files directly from **AmpliSeq.com**. **Note:** When you use this option, a target regions file and any available hotspot regions files specific for your panel are uploaded.	Click **AmpliSeq Import**.
Import a target regions file that you previously downloaded from **AmpliSeq.com** and saved to your local storage.	1. Under the **Target Regions** list, click **Upload**. 2. Click **Select file**, browse to, then select the target regions BED file, then click **Open**. 3. Select **Ion AmpliSeq™**, then click **Upload**.

4. Click **Next**.
5. In the **Annotation** step, accept the default settings, then click **Next**.
6. In the **Filters** step, accept the default settings, then click **Next**.
7. In the **Copy Number** step, select your panel-specific copy number baseline from the **Baseline** list, then click **Next**.
8. In the **Plugins** step, accept the default settings, then click **Next**.
9. In the **Final Report** step, select or confirm the final report template that is selected in the list, then click **Next**.
10. In the **Parameters** step, click **Next**.
11. In the **Confirm** step, name the analysis workflow, enter an optional description, then click **Save Workflow**.

The newly created analysis workflow is added to the list of analysis workflows in the **Workflows** tab in the **Overview** screen.

4 Procedural guidelines

Procedural guidelines

- Minimize freeze-thaw cycles of Oncomine™ tumor specific 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 11.
- Pipet viscous solutions, such as 5X Ion AmpliSeq™ HiFi Mix, FuPa Reagent, Switch Solution, DNA Ligase, and panels, slowly and ensure complete mixing by vortexing or pipetting up and down several times.
- Arrange samples in alternating columns on the plate for easier pipetting with multichannel pipettes during purification with the DynaMag™ Side Magnet.

Guidelines for RNA isolation, quantification, and input

- We recommend the MagMAX™ FFPE DNA/RNA Ultra Kit (Cat. No. A31881) for isolating RNA.
- We recommend the Qubit™ RNA HS Assay Kit (Cat. No. Q32855) for quantifying RNA.
- Treat total RNA with DNase before use.
- We recommend using 20 ng of total RNA for reverse transcription. Increasing the amount of total RNA will usually result in higher quality libraries, especially when RNA quality or quantity is unknown. With high-quality, well-quantified samples, as little as 1 ng total RNA can be used.
- In general, library yield from high quality RNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.

Guidelines for DNA isolation, quantification, and input

- We recommend the MagMAX™ FFPE DNA/RNA Ultra Kit (Cat. No. A31881) for isolating DNA.
- 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 spectrophotometry (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.
- We recommend using 20 ng (10 ng/pool) of DNA for manual library preparation or 10 ng of DNA for automated library preparation on an Ion Chef™ Instrument. Increasing the amount of DNA results in higher-quality libraries, especially when DNA quality or quantity is unknown.

Guidelines for library preparation

Before preparing libraries, we recommend planning how you will pool libraries for sequencing. Your desired sample throughput influences your choice of sequencing chip size, and the number of libraries prepared simultaneously. In addition, the sample type (DNA or RNA) and whether the libraries will be combined for simultaneous sequencing must be considered. This is particularly important for automated library preparation on the Ion Chef™ System because libraries come off the instrument normalized and pre-pooled in sets of eight libraries. We recommend preparing at least 6 positive template samples per Ion Chef™ Instrument run (no more than 2 no-template controls). For more information on combining libraries, see Chapter 8, “Combine and sequence libraries”.

Chip capacity

Chip type	Total reads per chip
Ion 530™ Chip	1.5–2.0 x 10^7
Ion 540™ Chip	6.0–8.0 x 10^7

We recommend an Average Read Depth of 2000 reads per DNA panel amplicon per barcode to achieve somatic variant calling at 5% allele frequency. We recommend 2 x 10^6 total reads per RNA panel barcode to achieve to ensure sufficeint read depth for fusion detection.

To calculate the number of DNA libraries from a given panel that a chip can accommodate use the following formula.

```
# DNA libraries = Total reads per chip/(# amplicons in the DNA panel x
desired Average Read Depth)
```

To calculate the number of RNA libraries that a chip can accommodate use the following formula.

```
# RNA libraries = Total reads per chip/2 x 10^6 Reads
```

To calculate the number of paired DNA and RNA libraries from the same sample that a chip can accommodate use the following formula.

```
# Paired libraries = Total reads per chip/(# amplicons in DNA panel x
desired Average Read Depth + 2 x 10^6 Reads per RNA library barcode)
```

5 Automated library preparation on the Ion Chef™ System

This chapter describes library preparation using the following components:

- Oncomine™ tumor specific panel
- Ion Torrent™ NGS Reverse Transcription Kit (Cat No. A45003)
- Ion AmpliSeq™ Kit for Chef DL8 (Cat No. A29024)

Automated RNA library preparation

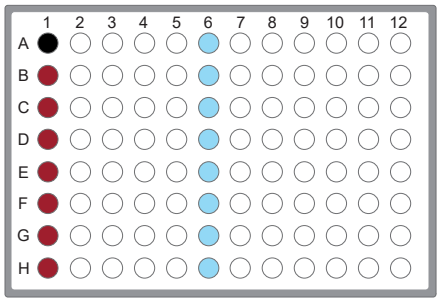
Reverse transcribe RNA for Chef Ready library preparation

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

1. Remove and discard the plate seal from an IonCode™ 96-well PCR Plate.
2. For each sample, add the following components into a single well in column 1 of the IonCode™ 96-well plate (provided in the Ion AmpliSeq™ Kit for Chef DL8). Prepare a master mix without sample RNA for multiple reactions.

Component	Volume
Ion Torrent™ NGS 5X Reaction Buffer	2 µL
Ion Torrent™ NGS 10X RT Enzyme Mix	1 µL
Total RNA (20 ng)[1]	≤7 µL
Nuclease-free Water	to 10 µL
Total volume per well	**10 µL**

[1] If preparing an RNA positive control sample along with high quality RNA samples, use 20 ng positive control sample input. If preparing an RNA positive control sample along with FFPE RNA samples, reduce the positive control sample input to 2 ng. Substitute an equal volume of nuclease-free water or low TE to prepare a no-template control (NTC).



- Column 1 wells contains a 10 µL reverse transcription reaction, or control reaction.
- *(Optional)* Positive control or Non template control (NTC)
- Each column 6 well contains a dried-down IonCode™ Barcode Adapter. The lowest barcode number is in A6, and the highest is in H6. All appear light blue in the actual plates.

Note:

- If you are processing fewer than 8 samples, it is preferable to add replicates or positive control samples to the run. Otherwise, pipet 15 µL of Nuclease-free Water as non-template control into column 1 wells that do not contain an RNA sample. We recommend placing controls in rows A & B.
- We recommend processing at least 6 samples per run. If processing 5 or fewer samples, we recommend that you quantify the output combined library by qPCR to ensure that an optimal concentration is used in templating reactions.

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. Briefly centrifuge the plate to collect any droplets at the bottom of the wells.

6. Pipet 5 µL of nuclease-free water into each cDNA synthesis reaction in column 1 of the IonCode™ 96-well plate.

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

Following completion of cDNA synthesis see "Thaw the reagents and prepare the instrument" in the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432) for instructions to prepare Oncomine™ tumor specific panel libraries on the Ion Chef™ System.

For information on how to set up the Ion Chef™ Instrument, see “Ion Chef™ Instrument setup information for automated RNA library preparation” on page 36.

Ion Chef™ Instrument setup information for automated RNA library preparation

See the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432) for detailed information on preparing libraries on the Ion Chef™ System.

During Ion Chef™ Instrument setup, enter the following parameters when prompted.

Stating material	# of primer pools	Target amplification cycles	Anneal & extension time
High quality RNA[1]	2	23	4 minutes
FFPE RNA[1]	2	29	4 minutes

[1] Due to the disparity in the required number of target amplification cycles for high quality and FFPE RNA we do NOT recommend running both high quality and FFPE samples on the same plate. If preparing a positive control (high quality) along with FFPE RNA samples, reduce the positive control sample input to 2 ng and use the FFPE cycling parameters.

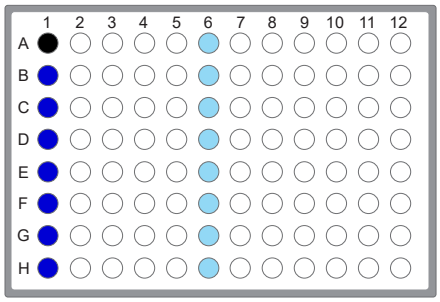
Automated DNA library preparation

Add sample DNA to an IonCode™ Barcode Adapters 96-well PCR plate

1. Remove, then discard the plate seal from an IonCode™ Barcode Adapters 96-well PCR plate.
2. For each sample, add the following components into a single well in column 1 of the IonCode™ Barcode Adapters 96-well PCR plate (provided in the Ion AmpliSeq™ Kit for Chef DL8).

Component	Volume
gDNA (10 ng, ≥0.67 ng/µL)[1]	≤15 µL
Nuclease-free Water	to 15 µL
Total volume per well	**15 µL**

[1] Substitute an equal volume of nuclease-free water or low TE to prepare a no-template control (NTC).



- Column 1 well contains 15 µL of diluted gDNA sample (0.67 ng/µL, 10 ng total), or Nuclease-free Water as non-template control.
- *(Optional)* Non template control (NTC)
- Each column 6 well contains a dried-down IonCode™ barcode. The lowest barcode number is in A6, and the highest is in H6. All appear light blue in the actual plates.

Note:

- If you are processing fewer than 8 samples, it is preferable to add replicates or positive control samples to the run. Otherwise, pipet 15 µL of Nuclease-free Water as non-template control into column 1 wells that do not contain a DNA sample.
- If processing 5 or fewer samples, we recommend that you quantify the output combined library by qPCR to ensure that an optimal concentration is used in templating reactions.

3. Carefully inspect each well for air bubbles. Remove any air bubbles by gentle pipetting. Alternatively, seal the plate with MicroAmp™ Adhesive Film, then briefly centrifuge the plate in a plate centrifuge.

IMPORTANT! Offset the film to the left so that the adhesive does not cover the barcode label. If the barcode label becomes damaged, you can override the error during Deck Scan on the Ion Chef™ Instrument.

Proceed to "Thaw the reagents and prepare the instrument" in the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432) for instructions to prepare Oncomine™ tumor specific panel libraries on the Ion Chef™ System.

For information on how to set up the Ion Chef™ Instrument, see “Ion Chef™ Instrument setup information for automated library preparation” on page 38.

Ion Chef™ Instrument setup information for automated library preparation

During Ion Chef™ Instrument setup, enter the following parameters when prompted.

Recommended number of amplification cycles for Oncomine™ tumor specific panels

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

[1] For automated library preparation on the Ion Chef™ Instrument, core Oncomine™ tumor specific panels that have not been customized through the addition or subtraction of gene targets should use these cycling parameters. The number of primer pairs in a pool is indicated on the tube label.

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

6 Manual library preparation

IMPORTANT! We recommend that you run a positive control the first time you prepare a library with each lot of Oncomine™ tumor specific panel.

RNA library preparation

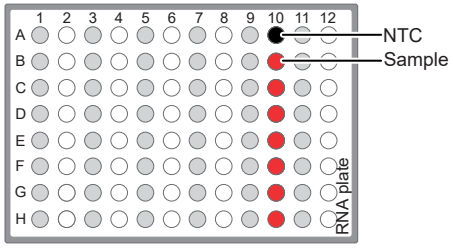
Use the components of the Ion Torrent™ NGS Reverse Transcription Kit (Cat. No. A45003) for the follow procedures.

Reverse transcribe RNA for manual library preparation

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 on ice or in a pre-chilled 4°C cold block. Prepare a master mix without sample RNA for multiple reactions.

Component	Volume
Ion Torrent™ NGS 5X Reaction Buffer	2 µL
Ion Torrent™ NGS 10X RT Enzyme Mix	1 µL
Total RNA (20 ng)[1]	≤7 µL
Nuclease-free Water	to 10 µL
Total volume per well	**10 µL**

[1] Substitute an equal volume of nuclease-free water or low TE to prepare a no-template control (NTC).



- *(Optional)* Non template control (NTC)
- RNA sample

3. 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.

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

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

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

5. Briefly centrifuge the plate to collect any droplets at the bottom of the wells, then proceed to the next step.

Prepare cDNA target amplification reactions

IMPORTANT! The cDNA synthesis reaction, primer pools, and 5X Ion AmpliSeq™ HiFi Mix are viscous. Pipet slowly and mix thoroughly.

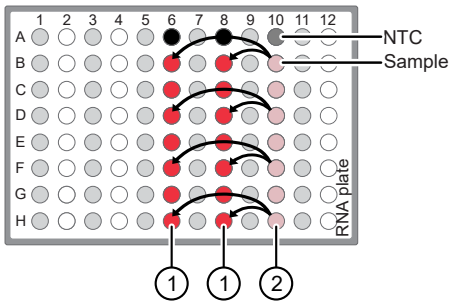
1. Place the 96-well plate in a pre-chilled cold block or on ice.

2. Thaw the 5X Ion AmpliSeq™ HiFi Mix on ice, gently vortex to mix, then briefly centrifuge to collect.

3. To each cDNA synthesis reaction add:

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

4. Mix by pipetting at least half the total volume up and down at least 5 times, then transfer 8 µL to each of two adjacent wells (~2 µL overage remainder).



● cDNA sample

● Non template control (NTC)

① 8 µL transferred cDNA target amplification reaction.

② ~2 µL cDNA target amplification reaction remaining.

5. Add 2 µL of 5X Oncomine™ tumor specific RNA panel primer pool-1 into the first well, then add 2 µL of primer pool-2 into the second well for a total of 10 µL in each well.

6. 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.

Proceed to "Amplify the cDNA targets" on page 41 .

Amplify the cDNA targets

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

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

2. Run the following program to amplify the target regions.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	99°C	2 min
Cycle; set number according to page 42	Denature	98°C	15 sec
	Anneal and extend	60°C	4 min (RNA Panel)
Hold	—	10°C	Hold

Recommended cycle number

Input nucleic acid[1]	Recommended number of cycles[2]	Cycle number adjustment[3]	
	10 ng RNA input	1 ng RNA input	100 ng RNA input
High quality RNA	22	+5	–3
FFPE RNA	28	+3	–3

[1] Due to the disparity in the required number of target amplification cycles for high quality and FFPE RNA we do NOT recommend running both high quality and FFPE samples on the same plate using the same input amount. If preparing a positive control (high quality) along with FFPE RNA samples, reduce the positive control sample input to 2 ng and use the FFPE cycling parameters.

[2] Number of cycles can be increased when input material quality or quantity is questionable.

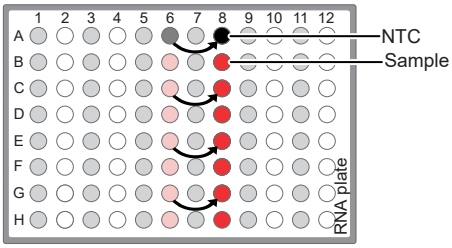
[3] The recommended number of cycles is based on 10 ng RNA input per primer pool. Adjust the cycle number for lower or higher RNA input.

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

Combine cDNA target amplification reactions

Note: If both cDNA and DNA samples use the same FuPa digestion conditions, samples may be combined onto a single plate for simultaneous processing.

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 “Partially digest the amplicons” on page 45.

DNA library preparation

Use the components of the Ion AmpliSeq™ Library Kit Plus (Cat. No. various) for the following procedures.

Prepare DNA target amplification reactions

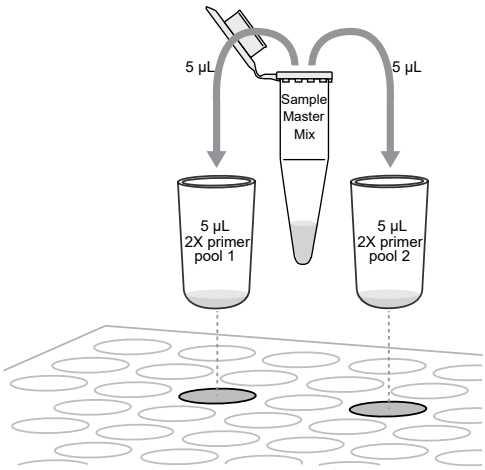
IMPORTANT! Primer pools and 5X Ion AmpliSeq™ HiFi Mix are viscous. Pipet slowly and mix thoroughly.

1. Place a 1.5-mL tube and 96-well plate on ice or in a pre-chilled 4°C cold block.
2. For each sample, prepare a target amplification master mix without primers in a 1.5-mL tube on ice.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	5 µL
DNA (20 ng)	≤7.5 µL
Nuclease-free Water	to 12.5 µL

3. Mix thoroughly by pipetting up and down 5 times, then transfer 5 µL of each sample-specific master mix to 2 wells of a 96-well PCR plate on ice or in a pre-chilled 4°C cold block.

   Note: When using multi block thermal cyclers ensure each pair of samples is in the same temperature zone for amplification. For example, use the same VeriFlex block on a Veriti™ Thermal Cycler.



4. Add 5 µL of 2X Oncomine™ tumor specific panel primer pool 1 to the first well, and 5 µL of primer pool 2 to the second well.
5. Seal the plate with MicroAmp™ Clear Adhesive Film.
6. Vortex for 5 seconds to mix, then briefly centrifuge to collect the contents. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

Proceed to “Amplify the targets” on page 44 .

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 [1]	**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] For manual library preparation, core Oncomine™ tumor specific panels that have not been customized through the addition or subtraction of gene targets should use these cycling parameters. The number of primer pairs in a pool is indicated on the tube label.

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.

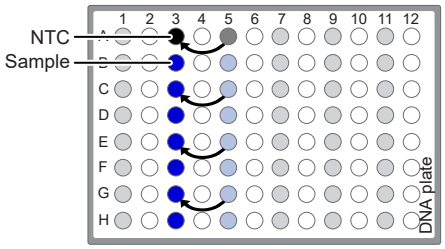
- When amplifying multiple samples in a single PCR plate, ensure that the input across the samples is roughly equivalent so that the selected cycle number for target amplification is optimal for all the samples in the run.

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

Combine DNA target amplification reactions

Note: If both DNA and cDNA samples use the same FuPa digestion conditions, samples may be combined onto a single plate for simultaneous processing.

1. Centrifuge the plate briefly, or tap the plate gently on a hard flat surface, to collect the contents at the bottom of the wells.
2. Carefully remove the plate seal.
3. For each sample, combine the two 10-µL target amplification reactions. The total volume for each sample should be approximately 20 µL.



Partially digest the amplicons

IMPORTANT! Keep each plate on ice or in a pre-chilled 4°C cold block while preparing the reactions.

Note: If preparing both DNA and cDNA samples and they use the same FuPa digestion conditions, samples may be combined onto a single plate for simultaneous processing. For more information, see “Transfer the DNA amplicons” on page 49.

1. Thaw the FuPa Reagent (brown cap) on ice, gently vortex to mix, then centrifuge briefly to collect.
2. Add 2 µL of FuPa Reagent to each amplified DNA or cDNA sample. The total volume per well is ~22 µL.
3. Seal each DNA or cDNA plate with a clear 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.

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

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

[1] Increase to 20 minutes for panels over 1,536 primer pairs. See the Oncomine™ tumor specific panel tube label for the number of amplicons.

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

Ligate adapters to the amplicons and purify

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

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

Ion Xpress™ Barcode Adapters require handling and dilution as described in "Ion Xpress™ Barcode Adapters only: Combine and dilute adapters".

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

Ion Xpress™ Barcode 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. Scale volumes as necessary. Use 2 µL of this barcode adapter mix in step 3 in "Perform the ligation reaction".

For example, combine the volumes indicated in the following table.

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. If you have not already done so, briefly centrifuge the plate to collect the contents, then carefully remove the plate seal.

3. 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™ Barcode Adapters, *or* diluted Ion Xpress™ barcode adapter mix (for barcoded libraries))	2 µL
3	DNA Ligase (blue cap)	2 µL
—	**Total volume** (including ~22 µL of digested amplicon)	**~30 µL**

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

5. Place a MicroAmp™ 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 unamplified library

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

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, move the plate side-to-side in the two positions of the magnet to wash the beads, then remove and discard the supernatant without disturbing the pellet.

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

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

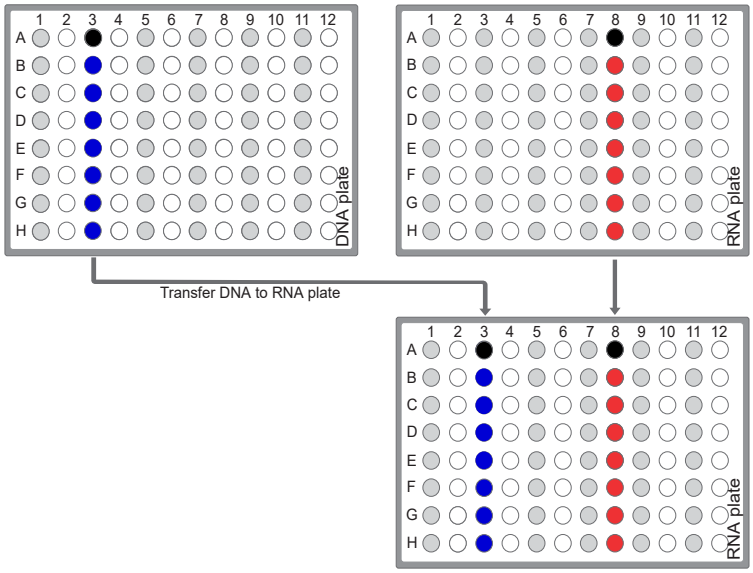
Transfer the DNA amplicons

If preparing both DNA and cDNA libraries, library preparations can be transferred to a single plate for simultaneous processing.

Note: If both DNA and cDNA samples use the same FuPa digestion conditions, samples may be combined onto a single plate for simultaneous digestion. For more information, see "Partially digest the amplicons" on page 45.

1. Remove the plate from the thermal cycler, then briefly centrifuge to collect the contents.
2. Carefully remove the adhesive film from the plate.

   IMPORTANT! Be careful when removing the film to minimize contamination.
3. Transfer the amplicons from the DNA plate to the corresponding empty wells of the RNA/cDNA plate.



- Sample DNA target amplification reactions
- Sample cDNA(RNA) target amplification reactions
- No template control (NTC) target amplification reaction

Elute and dilute the library

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

Proceed immediately to "Quantify the library by qPCR and calculate the dilution factor" on page 50.

Quantify the library by qPCR and calculate the dilution factor

Determine the concentration of each library by qPCR with the Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802) using the following steps. Analyze each sample, standard, and negative control in duplicate 20-µL reactions.

IMPORTANT! The following steps differ from those in the *Ion AmpliSeq™ Library Kit Plus User Guide*. Follow the steps below for quantifying your Oncomine™ tumor specific panel libraries.

1. Prepare three 10-fold serial dilutions of the *E. coli* DH10B Ion Control Library (~68 pM, from the Ion Library TaqMan™ Quantitation Kit) at 6.8 pM, 0.68 pM, and 0.068 pM. Mark these tubes as standards, then use these concentrations in the qPCR instrument software.
2. Use the volumes per reaction in the following table to prepare a PCR Reaction Mix for all reactions. We recommend duplicate reactions for each sample library, standard, and NTC. Include a 5–10% overage to accommodate pipetting errors.

Note: Volumes are provided per reaction. Double the volume for duplicate reactions.

Component	Volume per reaction	
	96-well plate	384-well plate
2X Ion Library qPCR Master Mix	10 µL	5 µL
Ion Library TaqMan™ Quantitation Assay, 20X	1 µL	0.5 µL
Total	**11 µL**	**5.5 µL**

3. In a MicroAmp™ Optical Reaction Plate, set up duplicate PCR reactions for each sample, standard, and NTC. To each well, add the following components:

Component	Volume per reaction	
	96-well plate	384-well plate
PCR Reaction Mix (from step 2)	11 µL	5.5 µL
1:100 dilution of the sample[1]	9 µL	4.5 µL

[1] Substitute E. coli DH10B standards prepared in step 1 for standards. Substitute nuclease-free water for NTC.

4. Seal the plate with a MicroAmp™ Optical Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.

5. Program your real-time instrument as follows:

Note: The fast cycling program was developed using the StepOnePlus™ Real-Time PCR System in Fast mode.

   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 (96-well plate) or 10 µL (384-well plate).

   d. Select FAM™ dye/MGB as the TaqMan™ probe reporter/quencher.

Reaction plate format	Run mode	Stage	Temperature	Time
96-well Standard OR 384-well Standard	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
48- / 96-well Fast OR 384-well Standard	Fast	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

6. Following qPCR, calculate the average concentration of the undiluted library by multiplying the determined concentration × 100.

7. Based on the calculated library concentration, determine the dilution that results in a concentration of ~50 pM for template preparation on the Ion Chef™ System.

   For example:

   - The undiluted library concentration is 300 pM.
   - The dilution factor is 300 pM/50 pM = 6.
   - Therefore, 10 µL of library that is mixed with 50 µL of Low TE (1:6 dilution) yields approximately 50 pM.

Note:

- Good results have been observed with libraries ≤50 pM. Proceed to the next step without further dilution.
- Libraries that yield significantly less than 50 pM can be rescued with library amplification. See "Tips" on page 93.

8. Proceed to Chapter 8, "Combine and sequence libraries" then template preparation, or store libraries as described below.

STOPPING POINT Libraries can be stored at 4–8°C for up to 1 month. For longer term, store at –20°C. We recommend transferring the supernatant to an RNase-free microcentrifuge tube for long-term storage. Alternatively, seal the plate with a new MicroAmp™ Clear Adhesive Film for long-term storage.

7 Create a Planned Run

- About Planned Runs 53
- Update Oncomine™ tumor specific panel templates in Torrent Suite™ Software 54
- Download and install BED files 54
- Create a Planned Run template for your Oncomine™ tumor specific panel 55
- Create a Planned Run 57

IMPORTANT! Before proceeding, we recommend that you update to the latest available versions of Torrent Suite™, Ion Reporter™, and Ion Chef™ System software. Contact your service representative for assistance with upgrading the software.

Before creating a Planned Run, you MUST upload the core panel- or custom panel-specific `<panel ID>_Oncomine_Designed.bed` file on the Ion Torrent™ Server. For more information, see "Download and install BED files" on page 54 . The core panel- and custom panel-specific `<panel ID>_Oncomine_Designed.bed` files are available for download from **AmpliSeq.com**.

Note: If the Ion Reporter™ account is not configured, configure it through Ion Reporter Configure settings (see "Configure the IonReporterUploader plugin in Torrent Suite™ Software" on page 101).

About Planned Runs

Planned Runs are digital instructions that are created in Torrent Suite™ Software for controlling the template preparation and sequencing instruments. Planned Runs contain settings such as number of flows, kit types, barcodes, sample information, and reference files (if any). Planned Runs are also used to track samples, chips, and reagents throughout the workflow, from template preparation on the Ion Chef™ Instrument through sequencing on an Ion GeneStudio™ S5 Series Sequencer and subsequent data analysis. Each chip that is prepared in an Ion Chef™ run requires its own Planned Run.

IMPORTANT! For more information on creating a Planned Run in Torrent Suite™ Software, including a complete description of each field in the **Create Plan** workflow bar, see the *Torrent Suite™ Software Help*, available by clicking the **Help** button in the software.

In Torrent Suite™ Software 5.14 or later, use the **Oncomine Tumor Specific DNA and Fusions** template as the primary Planned Run template for the Oncomine™ tumor specific panel. In Torrent Suite™ Software 5.12 use the Oncomine Tumor Specific DNA template as the primary Planned Run template for the Oncomine™ tumor specific panel.

Application	Torrent Suite™ Software template	Description
Oncology – Solid Tumor or DNA and Fusions	Oncomine Tumor Specific DNA and Fusions	DNA and RNA Planned Run template
	Oncomine Tumor Specific Fusions	RNA-only Planned Run template
Oncology – Solid Tumor or AmpliSeq DNA	Oncomine Tumor Specific DNA	DNA-only Planned Run template

Update Oncomine™ tumor specific panel templates in Torrent Suite™ Software

To install or update the Oncomine™ tumor specific panel templates, an off-cycle Torrent Suite™ Software update may be required. Contact your local service representative to schedule a software update.

1. Sign in to the Torrent Suite™ Software as an administrator.
2. In the upper right corner, click ⚙ **(Settings)** ▸ **Updates**, then scroll to the **Update Products** section.
3. In the **Name** column find Oncomine™ tumor specific panel, then in that row click **Update**.

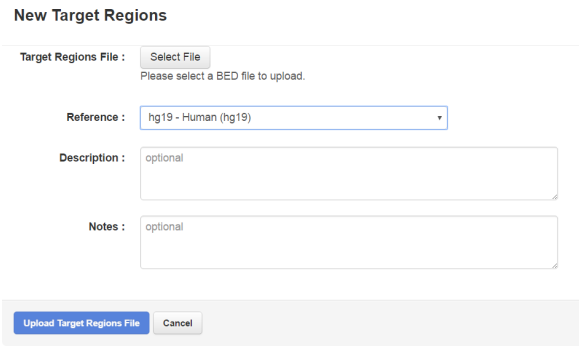
The software update begins automatically and displays as **Complete** when finished.

Download and install BED files

You must download the core panel- or custom panel-specific `<panel ID>_Oncomine_Designed.bed` file from **AmpliSeq.com** before proceeding. For more information, see “Order a custom Oncomine™ tumor specific panel” on page 19.

1. Extract the ZIP file containing the BED file to a location of your choice.
2. Sign in to the Ion Torrent™ Server on which you want to install the target regions and hotspots BED files.
3. Click the ⚙ ▾ **(Settings)** tab in the upper right of the screen, then select **References** from the dropdown list.
4. Upload the target regions BED file:
   a. In the left navigation menu, click **Target Regions**, then click the **Add Target Regions** button.
   b. Select **hg19 - Homo sapiens** from the **Reference** dropdown list.

c. Click **Select File**, then navigate to and select the target regions BED file, which has the following extension: `<panel ID>_Oncomine_Designed.bed`.



d. Click **Open**, then click **Upload Target Regions File**.

The installed BED file appears in the dropdown list on the Ion Torrent™ Server.

Create a Planned Run template for your Oncomine™ tumor specific panel

IMPORTANT! Before creating a Planned Run template, you need to import the `<panel ID>_Oncomine_Designed.bed` for your Oncomine™ tumor specific panel in to the Torrent Suite™ Software. For more information, see “Download and install BED files” on page 54.

Note: In Torrent Suite™ Software 5.12 or 5.14, before you can create a Planned Run template you must enable the **Oncomine Tumor Specific** Planned Run templates. For more information, see “Update Oncomine™ tumor specific panel templates in Torrent Suite™ Software” on page 54.

We recommend setting up a customized Planned Run template for reuse when the same conditions will be used for multiple runs. For more information about creating Planned Runs manually or from the generic application template, see the *Torrent Suite™ Software Help*. The **Oncomine Tumor Specific DNA** Planned Run template provided in the Torrent Suite™ Software includes all the information required for end to end templating, sequencing and data analysis of any of the core Oncomine™ tumor specific panels except the panel-specific `designed.bed` file.

However, to run a customized panel you must also first create:

- a copy number baseline preset specific to your Oncomine™ tumor specific panel. For more information see “Create a panel-specific CNV baseline” on page 27.
- an Ion Reporter™ Software analysis workflow specific to your Oncomine™ tumor specific panel.
- a custom Planned Run template in the Torrent Suite™ Software using the panel-specific `<panel ID>_Oncomine_Designed.bed` file.

1. Sign in to the Torrent Suite™ Software.
2. In the **Plan** tab, in the **Templates** screen, click **AmpliSeq DNA** in the left navigation menu.

3. In the list of templates, find **Oncomine Tumor Specific DNA**, then click ⚙ ▸ **Copy**.
   The **Copy Template** workflow opens to the **Save** step.

4. Enter or select the required information in each field:

Entry or selection [1]	Action
Template Name	Enter a name for the Planned Run template.
DNA Reference Library	Select **hg19(Human (hg19))**.
DNA Target Regions	Select your **<panel ID>_Oncomine_Designed.bed** file.

[1] DNA Hotspot Regions, Fusions Reference Library and Fusions Target Regions files are not necessary for analysis in Torrent Suite™ Software.

5. Under **Analysis Parameters**, confirm that **Default (Recommended)** is selected.

6. Click the **Ion Reporter** step, then select your Ion Reporter™ Software account (version 5.16 or later).

   Note: If the account is not configured, configure it through **Ion Reporter Configure** settings (see "Configure the IonReporterUploader plugin in Torrent Suite™ Software" on page 101). For instructions on how to install the IonReporterUploader plugin, see the *Torrent Suite™ Software Help*.

7. In the **Existing Workflow** dropdown list, select an Ion Reporter™ Software analysis workflow.

Panel type	Action
Core Oncomine™ tumor specific panel	Select the tumor-specific analysis workflow appropriate to the core panel being run.
Customized Oncomine™ tumor specific panel	Select the custom analysis workflow created for the specific panel being run.

8. Under **Sample Grouping** and **Ion Reporter Upload Options**, select **Self** and **Automatically upload to Ion Reporter after run completion** respectively, then click **Next**.

9. In the **Research Application** step, verify that the **Research Application** and **Target Technique** are set to **DNA** and **AmpliSeq DNA** respectively, then click **Next**.

10. In the **Kits** step, verify that the **Ion Chef** radio button is selected for the **Template Kit**, then confirm the following fields are completed as described:

Field	Selection	
	Manual library preparation	Automated library preparation
Instrument	Ion GeneStudio™ S5 System	
Library Kit Type	Ion AmpliSeq™ Library Kit Plus	Ion AmpliSeq™ Kit for Chef DL8
Template Kit	Select the **Ion Chef** radio button, then select the appropriate kit from the dropdown list. See the following table for kit selection information.	
Sequencing Kit	Ion S5™ Sequencing Kit	

(continued)

Field	Selection	
	Manual library preparation	Automated library preparation
Chip Type	Ion 530™ Chip or Ion 540™ Chip	
Barcode Set	Ion Dual Barcode Kit 1-96, IonCode Barcodes 1-32, or IonXpress	IonCode Barcodes 1–32
Flows	500	

Chip size used	Templating kit used
Ion 530™ Chip	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef
Ion 540™ Chip	Ion 540™ Kit – Chef

11. Select or edit the optional information fields appropriately for your run, then click **Next**.
12. In the **Plugins** step, confirm the **sampleID** and **coverageAnalysis** plugins are selected.
13. Configure the **coverageAnalysis** plugin as follows:
    a. Click **configure** next to **coverageAnalysis**.
    b. Ensure that the **Sample Tracking** checkbox is selected, then click **Save Changes**.
    c. Click **Next**.
14. In the **Projects** step, select the project appropriate to your run, then click **Next**.
15. In the **Save** step, click **Copy Template** to save the new Planned Run template.

The customized template is now available in the **Templates** screen, under the **AmpliSeq DNA** application.

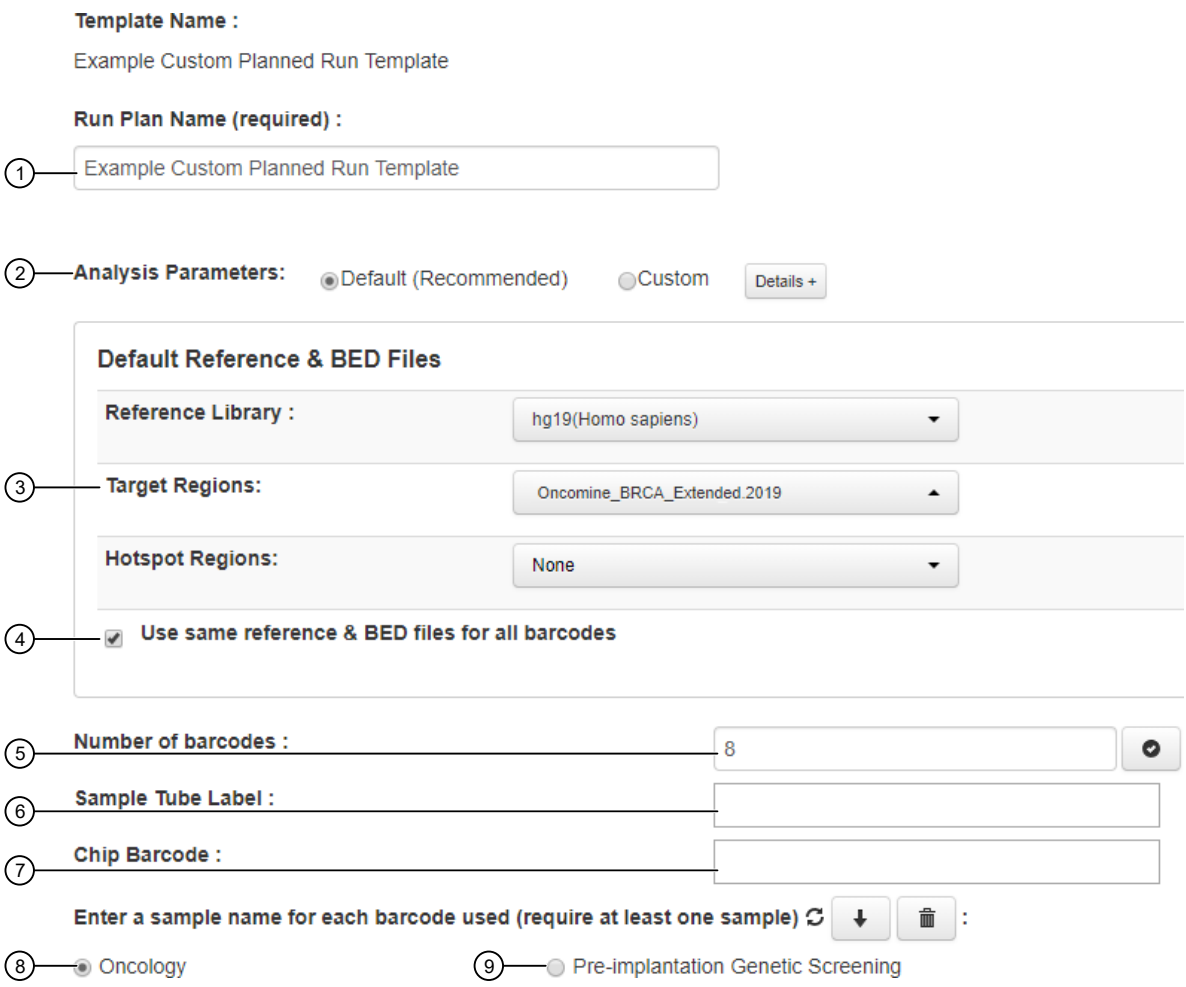
Create a Planned Run

1. Sign in to the Torrent Suite™ Software.
2. In the **Plan tab**, click **Templates**, then select **AmpliSeq DNA** in the left navigation menu.
3. Select the customized Planned Run template appropriate for the run.
   The **Create Plan** workflow bar opens to the **Plan** step.

4. Enter or select the following information.

Note: Row numbers in the table correspond to the callouts in the figure.

Callout	Field	Action
1	Run Plan Name	Enter a Planned Run name.
2	Analysis Parameters	Ensure the **Default (Recommended)** radio button is selected.
3	Target Regions	Select the **<panel ID>_Oncomine_Designed.bed** specific to the panel.
4	Use same reference & BED files for all barcodes	• Select the checkbox if sequencing multiple libraries prepared with the same assay. • Deselect the checkbox if sequencing multiple different assay libraries.
5	Number of barcodes	Enter the number of barcodes to be used in this run, then click ✓ to the right of this field. The default value is 8 barcodes.
6	Sample Tube Label	Enter or scan the barcode of the Ion Chef™ Library Sample Tube that will be used in the run.
7	Chip ID	No entry required.
8	Oncology	Ensure that the radio button is selected.
9	Pre-implantation Genetic Screening	Ensure that the radio button is deselected.



5. Enter sample information.

Note: Row numbers in the table correspond to the callouts in the figure.

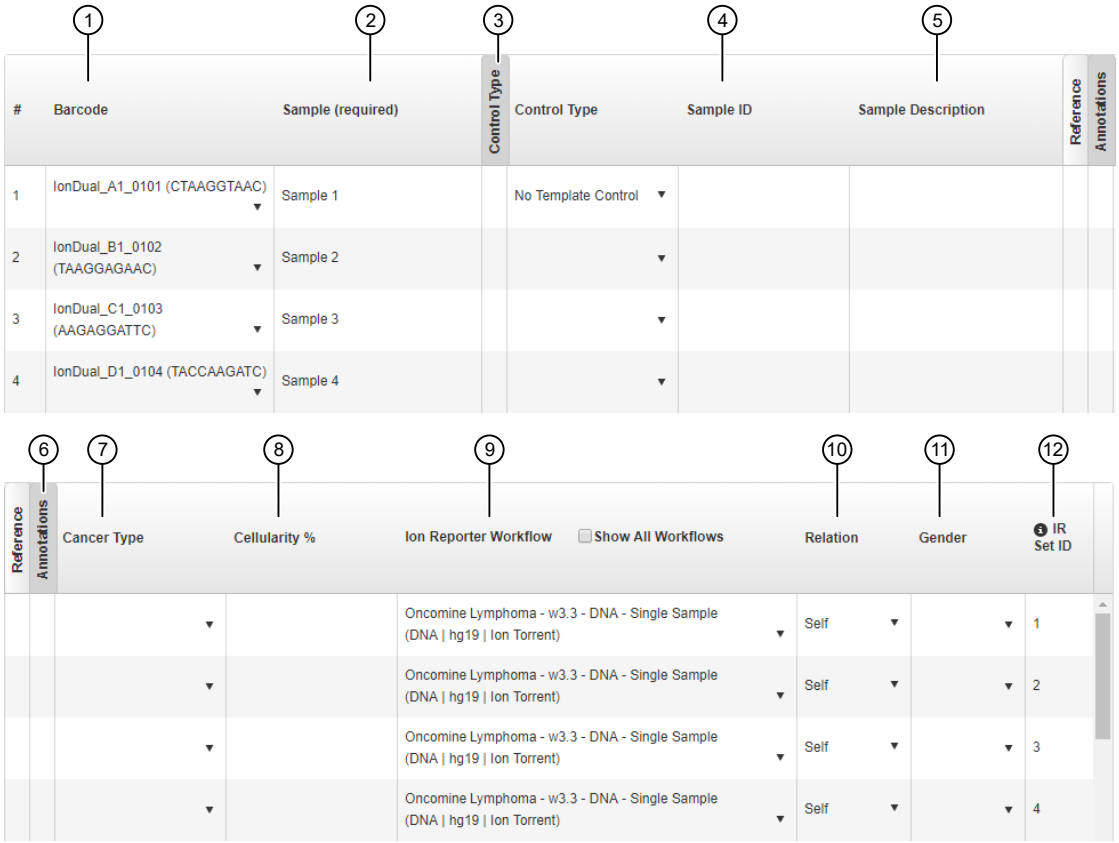
Callout	Field[1]	Action
1	Barcode	For each sample select the **Barcode** that will identify it from the dropdown list.
2	Sample Name	Accept the auto-populated sample names or click in a field, then enter a unique sample name. We recommend that the sample names (either auto-populated or user defined) that you pick are unique even between runs.
3	Control Type (expanded)	Select **No Template Control** from the dropdown list to designate a sample as a no template control.
4	Sample ID	*(Optional)* Click in the field, then enter a sample ID.
5	Sample Description	*(Optional)* Click in the field, then enter a sample description.
6	Annotations (expanded)	Click to reveal **Cancer Type** and **Cellularity %**.

(continued)

Callout	Field[1]	Action
7	Cancer Type	Select from the dropdown list. Click to copy the entry to all the rows.
8	Cellularity %	Enter a value. Click to copy the entry to all the rows. Required for copy number variant (CNV) determination.
9	Ion Reporter Workflow	Ensure the correct Ion Reporter™ Software analysis workflow is selected.
10	Relation	Ensure the correct value is auto-populated. Select from the dropdown list to change.
11	Gender	Select from the dropdown list. Click to copy the entry to all the rows.
12	IR Set ID[2]	The IR Set ID links individual samples for analysis. Ensure the correct value is auto-populated. Select from the dropdown list to change.

[1] Click vertical column headers (Control Type, Reference, Annotations) to reveal additional columns.

[2] Samples with the same IR Set ID are considered related samples and launched in the same analysis such as the DNA barcode and Fusions barcode of the same sample. Do not give unrelated samples the same IR Set ID value (even if that value is zero or blank).



6. Click **Plan Run**.

The run is listed in the **Planned Runs** screen under the name that you specified and is automatically used by the Ion Chef™ System when the associated Ion Chef™ Library Sample Tube is loaded on the instrument.

8 Combine and sequence libraries

- Guidelines for combining libraries 62
- Combine DNA libraries 63
- Combine RNA libraries 65
- Combine paired DNA and RNA libraries 66
- Guidelines for templating and sequencing 68

Guidelines for combining libraries

You can prepare barcoded libraries from different samples using Ion Torrent™ Dual, IonCode™ , or Ion Xpress™ Barcode Adapters. Multiple uniquely barcoded libraries of a single barcode type can be combined for simultaneous sequencing. We recommend combining fewer libraries initially and determining real limits empirically.

Oncomine™ tumor specific panel libraries, whether DNA or RNA, paired or unpaired, can be sequenced together on the same chip provided each has a unique barcode adapter of the same barcode type. Care must be taken when planning the sequencing run to ensure each library meets or exceeds the minimum number of reads required for variant detection. We do not recommend this combining strategy with automated library preparation due to the inefficent use of reagents required to prepare less than 8 libraries at a time.

Note: Libraries prepared on the Ion Chef™ Instrument have already been combined at 100 pM final concentration. Consideration should be given before automated library preparation to determine the number of libraries that are to be sequenced on the same chip. For more information, see “Guidelines for library preparation” on page 32.

To determine how many total libraries that can fit on a chip when combining unpaired and paired libraries from the same panel with unpaired and paired libraries from multiple additional panels we recommend determining the minimum number of reads that each individual or paired library requires first. Then calculate the number of each that fits on a chip together. When you have decided which libraries will be sequenced together determine the proportional volume of each library to combine to balance the number of reads across all libraries. See “Combine DNA libraries” on page 63, “Combine RNA libraries” on page 65, and “Combine paired DNA and RNA libraries” on page 66 for information on how to determine the number libraries for a given panel that can fit on a chip in each situation.

Recommended maximum number of individual Oncomine™ tumor specific panels per chip

Oncomine™ tumor specific panel or size (# genes)	DNA only		DNA + RNA	
	Ion 530™ Chip	Ion 540™ Chip	Ion 530™ Chip	Ion 540™ Chip
Oncomine™ tumor specific core panels (15–30)	8	32	4	16
Oncomine™ Homologous Recombination Repair Pathway Predesigned panel (28)	6	22	3	12
Oncomine™ tumor specific RNA panel	—	—	8[1]	32[1]
(1–10)	20	81	6	24
(11–30)	6	27	3	15
(31–50)	4	16	2	11
(51–75)	2	10	2	8
(76–100)	2	8	1	6
(101–150)	1	5	1	4

[1] RNA sample only.

Combine DNA libraries

You can prepare barcoded libraries from different samples using Ion Torrent™ Dual, IonCode™ , or Ion Xpress™ Barcode Adapters. Multiple uniquely barcoded libraries of a single barcode type can be combined for simultaneous sequencing. We recommend combining fewer libraries initially and determining real limits empirically.

Note: Libraries prepared on the Ion Chef™ Instrument have already been combined at 100 pM final concentration. Consideration should be given before automated library preparation to determine the number of libraries that are to be sequenced on the same chip. For more information, see "Guidelines for library preparation" on page 32.

- Combine DNA libraries prepared with the same Oncomine™ tumor specific panel.
  a. Determine the number of libraries prepared from your Oncomine™ tumor specific panel that can be combined based on the chip type being used.

Chip type	Sample libraries per chip[1]
Ion 530™ Chip	= 1.5–2.0 x 10^7/(# amplicons in panel x desired Ave Read Depth)[2]
Ion 540™ Chip	= 6.0–8.0 x 10^7/(# amplicons in panel x desired Ave Read Depth)[1]

[1] We recommend rounding down when determining how many libraries to combine on a single chip.
[2] We recommend an average read depth of 2000 to achieve somatic variant calling at 5% allele frequency.

b. Dilute each individual library to 50-pM concentration.

c. Add an equal volume of each library to a single tube such that the total volume of all combined libraries is ≥30 µL. 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.

Note: Do not pipet volumes <2 µL to minimize pipeting errors.

- Combine DNA libraries prepared from multiple Oncomine™ tumor specific panels.

a. Determine the number of reads that each individual library requires, then determine how many of each library can be combined based on the chip type being used. In this example the Oncomine™ Homologous Recombination Repair Pathway Predesigned core panel (1481 amplicons, panel A) and Oncomine™ Colorectal and Pancreatic core panel (898 amplicons, panel B) with a desired average read depth of 2000 are used.

Panel	Number of reads required per library[1]
Panel A	= (# amplicons in HRR panel x desired Ave Read Depth) = (1481 x 2000) = 2.962×10^6
Panel B	= (# amplicons in CRP panel x desired Ave Read Depth) = (898 x 2000) = 1.796×10^6

[1] We recommend an average read depth of 2000 to achieve somatic variant calling at 5% allele frequency.

Chip type	Sample libraries per chip[1]
Ion 530™ Chip	= $1.5–2.0 \times 10^7$/(# reads required for HRR panel) = $1.5–2.0 \times 10^7 / 2.962 \times 10^6$ = 5.0–6.7 = $1.5–2.0 \times 10^7$/(# reads required for CRP panel) = $1.5–2.0 \times 10^7 / 1.796 \times 10^6$ = 8.3–11.1
Ion 540™ Chip	= $6.0–8.0 \times 10^7$/(# reads required for HRR panel) = $6.0–8.0 \times 10^7 / 2.962 \times 10^6$ = 20.3–27.0 = $6.0–8.0 \times 10^7$/(# reads required for CRP panel) = $6.0–8.0 \times 10^7 / 1.796 \times 10^6$ = 33.4–44.5
Example: Ion 530™ Chip, 4 Oncomine™ Homologous Recombination Repair Pathway Predesigned libraries and fill the remaining space with Oncomine™ Colorectal and Pancreatic libraries	
= $1.5–2.0 \times 10^7 - 4(2.962 \times 10^6) = 3.152–8.152 \times 10^6$ available reads X Oncomine™ Colorectal and Pancreatic libraries = $3.152–8.152 \times 10^6 / 1.796 \times 10^6$ = 1.7–4.5 You can accommodate 4 Oncomine™ Homologous Recombination Repair Pathway Predesigned libraries and 2–4 Oncomine™ Colorectal and Pancreatic libraries on an Ion 530™ Chip.	

[1] We recommend rounding down when determining how many libraries to combine on a single chip.

b. Dilute each individual libraries to 50-pM concentration.

c. Calculate the volume of each panel A library and each panel B library to be combined for equal coverage of each library in a total volume of 50 µL. Using the example above the formula is:

- Volume of each Panel A library = $50\ \mu L \times \frac{\text{Number of reads per Panel A library}}{\text{Total number of reads per chip}}$
- Volume of each Panel B library = $50\ \mu L \times \frac{\text{Number of reads per Panel B library}}{\text{Total number of reads per chip}}$

Example:

Volume of each library from Panel A = 50 µL × ($2.962 \times 10^6 / 2.0 \times 10^7$) = 7.4 µL

Volume of each library from Panel B = 50 µL × ($1.796 \times 10^6 / 2.0 \times 10^7$) = 4.5 µL

Total volume of combined libraries ~50 µL = (4 x 7.4) + (4 x 4.5) = 47.6 µL

Note: We recommend pipeting volumes ≥2 µL to minimize pipeting errors. If needed increase the smallest calculated volume to 2 µL and proportionally increase all other volumes rather than pipet <2 µL.

d. Add the calculated volume of each of the libraries to a single tube. 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.

Proceed to "Guidelines for templating and sequencing" on page 68.

Combine RNA libraries

You can prepare barcoded libraries from different samples using Ion Torrent™ Dual, IonCode™ , or Ion Xpress™ Barcode Adapters. Multiple uniquely barcoded libraries of a single barcode type can be combined for simultaneous sequencing. We recommend combining fewer libraries initially and determining real limits empirically.

Note: Libraries prepared on the Ion Chef™ Instrument have already been combined at 100 pM final concentration. Consideration should be given before automated library preparation to determine the number of libraries that are to be sequenced on the same chip. For more information, see "Guidelines for library preparation" on page 32.

1. Determine the number of libraries prepared from the Oncomine™ tumor specific RNA panel that can be combined based on the chip type being used.

Chip type	Sample libraries per chip[1]	Maximum number of libraries
Ion 530™ Chip	= 1.5–2.0 x 10^7/ 2.0 x 10^6 reads per barcode[2]	8
Ion 540™ Chip	= 6.0–8.0 x 10^7/ 2.0 x 10^6 reads per barcode[2]	32

[1] We recommend rounding down when determining how many libraries to combine on a single chip.

[2] We recommend a minimum of 2.0 x 10^6 total reads per barcode to achieve somatic variant calling at 5% allele frequency.

2. Dilute each individual libraries to 50-pM concentration.

3. Add an equal volume of each library to a single tube such that the total volume of all combined libraries is ≥30 µL. 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.

Note: Do not pipet volumes <2 µL to minimize pipeting errors.

Proceed to "Guidelines for templating and sequencing" on page 68.

Combine paired DNA and RNA libraries

You can prepare barcoded libraries from different samples using Ion Torrent™ Dual, IonCode™ , or Ion Xpress™ Barcode Adapters. Multiple uniquely barcoded libraries of a single barcode type can be combined for simultaneous sequencing. We recommend combining fewer libraries initially and determining real limits empirically.

Note: Libraries prepared on the Ion Chef™ Instrument have already been combined at 100 pM final concentration. Consideration should be given before automated library preparation to determine the number of libraries that are to be sequenced on the same chip. For more information, see "Guidelines for library preparation" on page 32.

- Combine paired DNA and RNA libraries prepared from the same sample with a single Oncomine™ tumor specific panel.

1. Determine the number of paired DNA + RNA libraries prepared from your Oncomine™ tumor specific panel and the Oncomine™ tumor specific RNA panel that can be combined based on the chip type being used.

Chip type	Paired sample libraries per chip[1]
Ion 530™ Chip	= 1.5–2.0 x 10^7/(# amplicons in DNA panel x Desired Read Depth + 2 x 10^6 Reads per RNA library barcode)[2]
Ion 540™ Chip	= 6.0–8.0 x 10^7/(# amplicons in DNA panel x Desired Read Depth + 2 x 10^6 Reads per RNA library barcode)
Example: Ion 540™ Chip, Oncomine™ Bladder core panel (887 amplicons)	
= 6.0–8.0 x 10^7/(887 x 2000 + 2 x 10^6) = 15.9–21.2	

[1] We recommend rounding down when determining how many libraries to combine on a single chip.

[2] We recommend an average read depth of 2000 per DNA panel amplicon and 2 x 10^6 for each RNA library barcode to achieve somatic variant calling at 5% allele frequency.

2. Dilute each individual libraries to 50-pM concentration.

3. Calculate the volume of each DNA library and each RNA library to be combined for equal coverage of each library in a total volume of 50 µL.

   - Volume of each DNA library = $50\ \mu L \times \frac{\text{Number of reads per DNA library}}{\text{Total number of reads per chip}}$
   - Volume of each RNA library = $50\ \mu L \times \frac{\text{Number of reads per RNA library}}{\text{Total number of reads per chip}}$

   Example:

   Volume of each DNA library = 50 µL × (887 x 2000/6.0 x 10^7) = 1.5 µL

   Volume of each RNA library = 50 µL × (2.0 x 10^6/6.0 x 10^7) = 1.7 µL

   Total volume of combined libraries ~50 µL = (16 x 1.5) + (16 x 1.7) = 51.2 µL

   Note: We recommend pipeting volumes ≥2 µL to minimize pipeting errors. If needed increase the smallest calculated volume to 2 µL and proportionally increase all other volumes rather than pipet <2 µL.

   Example:

   2/1.5 = 1.33

   1.33 x 1.7 = 2.3

   Total volume of combined libraries = (16 x 2.0) + (16 x 2.3) = 68.8 µL

4. Add the calculated volume for each library to a single tube. 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.

Proceed to "Guidelines for templating and sequencing" on page 68.

Guidelines for templating and sequencing

Proceed to template preparation and sequencing using the following kits.

Chip	Template System	Sequencer	Kit	User Guide
Ion 530™ Chip	Ion Chef™ System	Ion S5™ XL Sequencer, Ion GeneStudio™ S5 Plus Sequencer, or Ion GeneStudio™ S5 Prime Sequencer	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef (Cat. Nos. A34019 or A34461[1])	*Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide* (Pub. No. MAN0016854)
Ion 540™ Chip			Ion 540™ Kit – Chef (Cat. No. A30011)	*Ion 540™ Kit – Chef User Guide* (Pub. No. MAN0010851)

[1] Cat. No. A34461 only provides sufficient reagents for a single sequencing run per initialization when performing 400 bp read sequencing.

To create a specific Run Plan for use in templating and sequencing see Chapter 7, “Create a Planned Run”. Refer to the appropriate user guide listed in the table for more information.

9 Variant analysis

- Analysis workflows in Ion Reporter™ Software 5.16 69
- Analysis workflows in Ion Reporter™ Software 5.14 72
- Manually launch an analysis 74
- View results 75
- Oncomine™ BRCA analysis results 83
- Generate an Analysis Results Report 90
- Download Ion Reporter™ annotation VCF or TSV files 91

IMPORTANT! We recommend updating to the latest available version of Ion Reporter™ Software. To analyze Oncomine™ tumor specific panel data using Ion Reporter™ Software 5.16, you must have a software package installed by your service representative.

Analysis workflows in Ion Reporter™ Software 5.16

If you selected the appropriate Ion Reporter™ Software analysis workflow when setting up your Planned Run in Torrent Suite™ Software, automated analysis has already been performed and you can view the Oncomine™ analysis results in Ion Reporter™ Software. For instructions on manually launching an analysis, see "Manually launch an analysis" on page 74.

Note: Microsoft™ Excel™, or other spreadsheet tool, is required for viewing VCF, CSV, and TSV files.

Ion Reporter™ Software 5.16 or later includes the analysis workflows listed in the following table. For panel-specific custom Oncomine™ tumor specific panel analysis workfows, see "Create an Ion Reporter™ Software analysis workflow for your predesigned or custom Oncomine™ tumor specific panel" on page 25. Oncomine™ tumor specific panel analysis workflows are for Research Use Only. Not for use in diagnostic procedures.

Ion Reporter™ Software 5.16 analysis workflow name[1]	Description
Oncomine Lymphoma - 530 - w4.1 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries from the Oncomine™ Lymphoma assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Lymphoma - 540 - w4.1 - DNA - Single Sample	
Oncomine BRCA Expanded - 530 - w4.1 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries from the Oncomine™ BRCA Expanded assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or

(continued)

Ion Reporter™ Software 5.16 analysis workflow name[1]	Description
Oncomine BRCA Expanded - 540 - w4.1 - DNA - Single Sample	Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine BRCA Expanded - 530 - w4.1 - DNA and Fusions - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries, as well as gene fusions from targeted RNA libraries, from the Oncomine™ BRCA Expanded assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine BRCA Expanded - 540 - w4.1 - DNA and Fusions - Single Sample	
Oncomine Bladder - 530 - w4.1 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries from the Oncomine™ Bladder assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Bladder - 530 - w4.1 - DNA - Single Sample	
Oncomine Bladder - 530 - w4.1 - DNA and Fusions - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries, as well as gene fusions from targeted RNA libraries, from the Oncomine™ Bladder assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Bladder - 540 - w4.1 - DNA and Fusions - Single Sample	
Oncomine Kidney - 530 - w4.1 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries from the Oncomine™ Kidney assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Kidney - 540 - w4.1 - DNA - Single Sample	
Oncomine Kidney - 530 - w4.1 - DNA and Fusions - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries, as well as gene fusions from targeted RNA libraries, from the Oncomine™ Kidney assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Kidney - 540 - w4.1 - DNA and Fusions - Single Sample	
Oncomine Melanoma - 530 - w4.1 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries from the Oncomine™ Melanoma assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Melanoma - 540 - w4.1 - DNA - Single Sample	
Oncomine Melanoma - 530 - w4.1 - DNA and Fusions - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries, as well as gene fusions from targeted RNA libraries, from the Oncomine™ Melanoma assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Melanoma - 540 - w4.1 - DNA and Fusions - Single Sample	
Oncomine Prostate - 530 - w4.1 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries from the Oncomine™ Prostate assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™

(continued)

Ion Reporter™ Software 5.16 analysis workflow name[1]	Description
Oncomine Prostate - 540 - w4.1 - DNA - Single Sample	Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Prostate - 530 - w4.1 - DNA and Fusions - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries, as well as gene fusions from targeted RNA libraries, from the Oncomine™ Prostate assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Prostate - 540 - w4.1 - DNA and Fusions - Single Sample	
Oncomine Gynecological - 530 - w4.1 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries from the Oncomine™ Gynecological assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Gynecological - 540 - w4.1 - DNA - Single Sample	
Oncomine Gynecological - 530 - w4.1 - DNA and Fusions - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries, as well as gene fusions from targeted RNA libraries, from the Oncomine™ Gynecological assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Gynecological - 540 - w4.1 - DNA and Fusions- Single Sample	
Oncomine Gastric and Esophageal - 530 - w4.1 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries from the Oncomine™ Gastric and Esophageal assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Gastric and Esophageal - 540 - w4.1 - DNA - Single Sample	
Oncomine Gastric and Esophageal - 530 - w4.1 - DNA and Fusions - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries, as well as gene fusions from targeted RNA libraries, from the Oncomine™ Gastric and Esophageal assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Gastric and Esophageal - 540 - w4.1 - DNA and Fusions - Single Sample	
Oncomine Colorectal and Pancreatic - 530 - w4.1 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries from the Oncomine™ Colorectal and Pancreatic assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Colorectal and Pancreatic - 540 - w4.1 - DNA - Single Sample	
Oncomine Colorectal and Pancreatic - 530 - w4.1 - DNA and Fusions - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries, as well as gene fusions from targeted RNA libraries, from the Oncomine™ Colorectal and Pancreatic assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Colorectal and Pancreatic - 540 - w4.1 - DNA and Fusions - Single Sample	

(continued)

Ion Reporter™ Software 5.16 analysis workflow name[1]	Description
Oncomine Liver - 530 - w4.1 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries from the Oncomine™ Liver assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Liver - 540 - w4.1 - DNA - Single Sample	
Oncomine Liver - 540 - w4.1 - DNA and Fusions - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries, as well as gene fusions from targeted RNA libraries, from the Oncomine™ Liver assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.
Oncomine Liver - 540 - w4.1 - DNA and Fusions - Single Sample	
Oncomine Tumor Specific - w4.1 - Fusions - Single Sample	Detects and annotates gene fusions from targeted RNA libraries from the Oncomine™ tumor specific RNA panel (using Ion AmpliSeq technology) run on the Ion 530™ Chip or Ion 540™ Chip respectively. Released with: Ion Reporter™ Software 5.16. Workflow Version: 4.1.

[1] The workflows listed in this table are available in Ion Reporter™ Software 5.16. Analysis workflow names can vary depending on the version of the Ion Reporter™ Software used.

Analysis workflows in Ion Reporter™ Software 5.14

In Ion Reporter™ Software 5.14, the Oncomine™ BRCA Expanded analysis workflow now includes end to end coverage of both BRCA1 and BRCA2 genes with optimized variant calling for SNV, long deletions, exon deletion, exon duplication, and whole gene somatic deletion variants. Any Oncomine™ tumor specific panel that includes coverage of both the BRCA1 and BRCA2 genes can now be analyzed using a custom copy/edit version of the Oncomine™ BRCA Expanded analysis workflow on either an Ion 530™ or Ion 540™ Chip. Additionally, the Oncomine™ Lymphoma- 540 - w4.0 - DNA - Single Sample analysis workflow can be copy/edited to create a custom analysis workflow for any core or custom Oncomine™ tumor specific panel allowing sequencing and analysis on an Ion 540™ Chip.

If you selected the appropriate Ion Reporter™ Software analysis workflow when setting up your Planned Run in Torrent Suite™ Software, automated analysis has already been performed and you can view the Oncomine™ analysis results in Ion Reporter™ Software. For instructions on manually launching an analysis, see “Manually launch an analysis” on page 74.

Note: Microsoft™ Excel™, or other spreadsheet tool, is required for viewing VCF, CSV, and TSV files.

Ion Reporter™ Software 5.14 or later includes the analysis workflows listed in the following table. Oncomine™ tumor specific panel analysis workflows are for Research Use Only. Not for use in diagnostic procedures.:

Ion Reporter™ Software 5.14 analysis workflow name[1]	Description
Oncomine BRCA Expanded - 530 - w4.0 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries from the Oncomine™ BRCA Expanded assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip. Released with: Ion Reporter™ Software 5.14. Workflow Version: 4.0.
Oncomine BRCA Expanded - 540 - w4.0 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries from the Oncomine™ BRCA Expanded assay (using Ion AmpliSeq™ technology) run on the Ion 540™ Chip. Released with: Ion Reporter™ Software 5.14. Workflow Version: 4.0.
Oncomine Lymphoma - 530 - w4.0 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries from the Oncomine™ Lymphoma assay (using Ion AmpliSeq™ technology) run on the Ion 530™ Chip. Released with: Ion Reporter™ Software 5.14. Workflow Version: 4.0.
Oncomine Lymphoma - 540 - w4.0 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries, as well as gene fusions from targeted RNA libraries, from the Oncomine™ Lymphoma assay (using Ion AmpliSeq™ technology) run on the Ion 540™ Chip. Released with: Ion Reporter™ Software 5.14. Workflow Version: 4.0.

[1] The workflows listed in this table are available in Ion Reporter™ Software 5.14. Analysis workflow names can vary depending on the version of the Ion Reporter™ Software used.

Custom Ion Reporter™ Software 5.14 analysis workflows for use with Oncomine™ tumor specific panels

To analyze a core or custom Oncomine™ tumor specific assay for which an Ion Reporter™ Software 5.14 analysis workflow is not provided, you must create a custom analysis workflow for use with your panel. First copy one of the preinstalled analysis workflow templates, then add panel-specific target regions files, and any available hotspots files or CNV baseline. For RNA samples, you must also add fusion panel files to your custom analysis workflow.

IMPORTANT! We recommend that you use the provided Oncomine™ BRCA Expanded and Oncomine™ Lymphoma analysis workflows for analyzing core or customized Oncomine™ BRCA Expanded and Oncomine™ Lymphoma tumor specific panels instead of creating a custom analysis workflow.

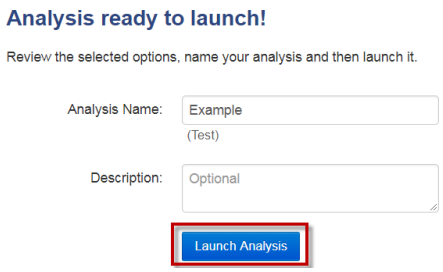
- For any panel containing the BRCA1 and BRCA2 genes, copy/edit the Oncomine™ BRCA Expanded analysis workflow.
- To create an Ion 540™ Chip analysis workflow for any Oncomine™ tumor specific panel other than Oncomine™ BRCA Expanded or Oncomine™ Lymphoma, copy/edit the Oncomine™ Lymphoma - 540 - w4.0 - DNA - Single Sample analysis workflow.

For details on creating custom analysis workflows in Ion Reporter™ Software, see “Create an Ion Reporter™ Software analysis workflow for your predesigned or custom Oncomine™ tumor specific panel” on page 25 or the *Ion Reporter™ Software 5.14 User Guide* (Pub. No. MAN0019036).

After you create the custom analysis workflow, you can select the analysis workflow in Torrent Suite™ Software to transfer data automatically to the appropriate Ion Reporter™ Server and use the analysis workflow for the data analysis in the Ion Reporter™ Software.

Manually launch an analysis

1. Sign in to the Ion Reporter™ Software.
2. In the **Workflows** tab, in the **Overview** screen, select **DNA and Fusions** from the **Research Application** dropdown list.
3. *(Optional)* In Search, enter a term, then click **Go** (or press Enter).
4. In the **Workflow Name** column, click the appropriate workflow (for example, Oncomine BRCA Expanded - 530 - w4.1 - DNA - Single Sample), then click **Actions** ▸ **Launch Analysis** in the **Details** pane.
5. In the **Samples** step, search by any unique identifier you used to label your samples during setup, then ensure the sample **Cellularity %** and **Sample Type** are defined.
6. Click the checkbox to select a DNA sample and a Fusions sample.
7. In the **Sample Groups** pane, click **Add Samples** to add the selected samples to a sample group.
8. Enter a **Group Name**, click **Add to Analysis**, then click **Next**.
9. In the **Plugins** step, ensure that the **Oncomine™ Variant Annotator 3.1** plugin is selected, then click **Next**.
10. *(Optional)* Enter an **Analysis Name** and **Description**.
11. Click **Launch Analysis**.



View results

Ion Reporter™ Software analyses are performed automatically on uploading of the data files from the Torrent Suite™ Software.

1. Sign in to the Ion Reporter™ Software.

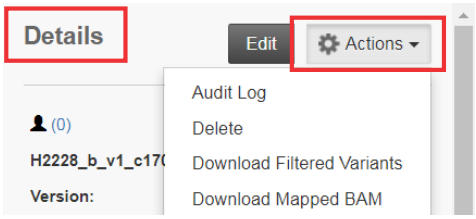
2. Click the **Analyses** tab.
   The **Overview** screen displays a list of analyses in the **Analyses** table.

3. *(Optional)* Filter the **Analyses** table.

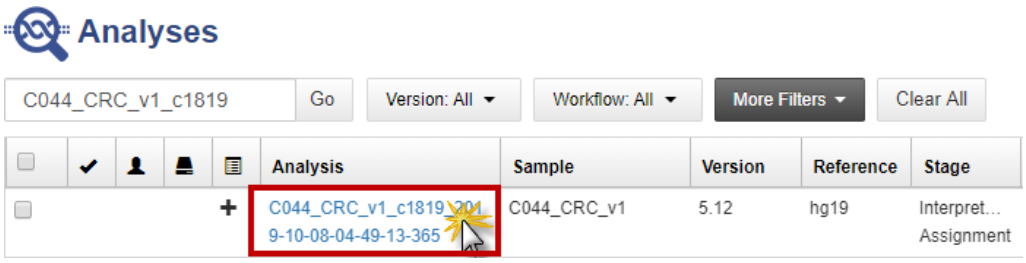
   - In the **Overview** screen, click **More Filters** ▸ **Research Application**. In the **Research Application** dropdown list, select the Oncomine™-specific analyses (**DNA**, **Fusions**, **DNA and Fusions**, or **Annotate Variants**).
   - Enter a search term in the search field, then click **Go** (or press **Enter**).

   To further refine the list of analyses, apply additional filters from the **More Filters** dropdown list or click the column headers. The **Analyses** table automatically updates based on the filter selections and search term.

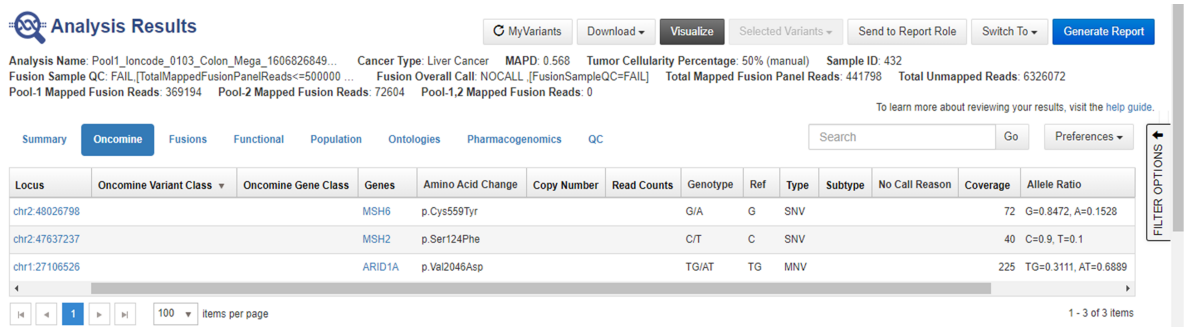
4. Click on a row (but not on the sample data set hyperlink) to open the **Details** pane on the right side of the screen for that analysis.
   In the **Details** pane, you can view **Workflow Details** and access the **Actions** dropdown list.



5. Click a sample result hyperlink in the **Analysis** column to open the **Analysis Results** page.



The **Analysis Results** page opens to the **Oncomine™** tab, and displays only variants relevant to cancer.



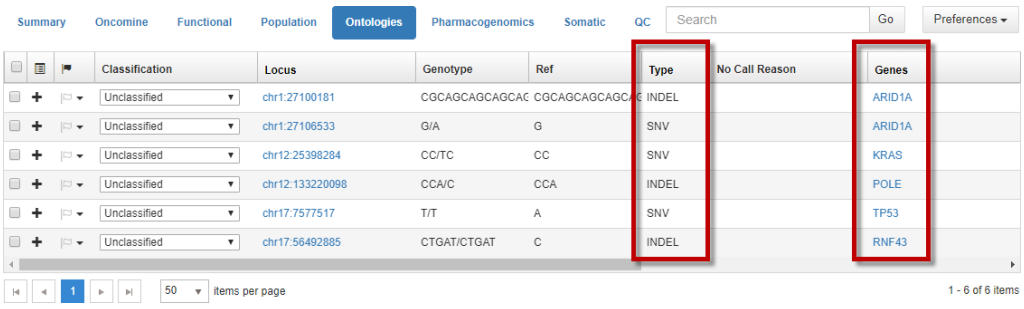
Item	Description
MAPD (Median Absolute Pairwise Difference)	The MAPD metric is a measure of read coverage noise detected across all amplicons in a panel. Higher MAPD typically translates to lower coverage uniformity. Lower coverage uniformity can result in missed or erroneous CNV calls. MAPD score is viewable in downloadable VCF file or review of the Analysis Results of a single sample extended analysis. To make a CNV call the following criteria must be met: • MAPD <0.4 • P-value <10-5 • CNV Ratio for a copy number gain must be >2 • CNV Ratio for a copy number loss must be <0.85
Tumor Cellularity Percentage	The percent of tumor cells in the sample.

6. In the **Analysis Results** table, sort or filter the data using the Oncomine™-specific annotations. See the Ion Reporter™ Software help menu for more options.
   a. In the **Filter Options** pane, select the desired **Filter Chain**.

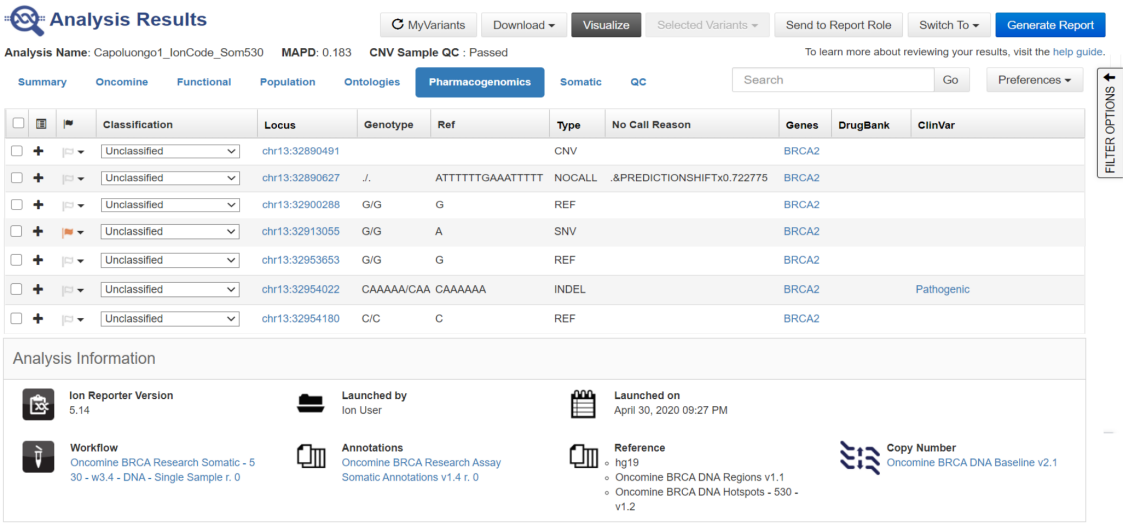
   Note:
   - The default **Filter Chain** is **Oncomine™ Extended**, which limits the results that are displayed to variants relevant to cancer research only. Each variant that is called must meet all the conditions of the filter chain to be filtered-in. For more information on filter chains, see the *Ion Reporter™ Software Help*.
   - Select **No Filter** to view all the variant calls attempted by the variant caller.
   - Saving the analysis using a filter chain other than **Oncomine™ Extended** changes the variant calls that are saved in the VCF file and can affect downstream workflows.

   b. In the **Oncomine™** tab, click the column headers to sort the list of variants by **Oncomine Variant Class** or **Oncomine Gene Class**.

   c. In the **Ontologies** tab, click the column headers to sort the list by variant **Type** or **Genes**.



d. Click **Pharmacogenomics** to view the **ClinVar** column. Click the link in the **ClinVar** column for a selected variant to open an NCBI ClinVar website where information about the ClinVar variant annotation is available.



After you review, filter, and sort your Analysis Result, you can create a report (see "Generate an Analysis Results Report" on page 90), or download the analysis files (see "Download Ion Reporter™ annotation VCF or TSV files" on page 91). You can view the extracted files individually, or upload a VCF file to a software that requires VCF files, such as Ion Torrent™ Oncomine™ Reporter Software.

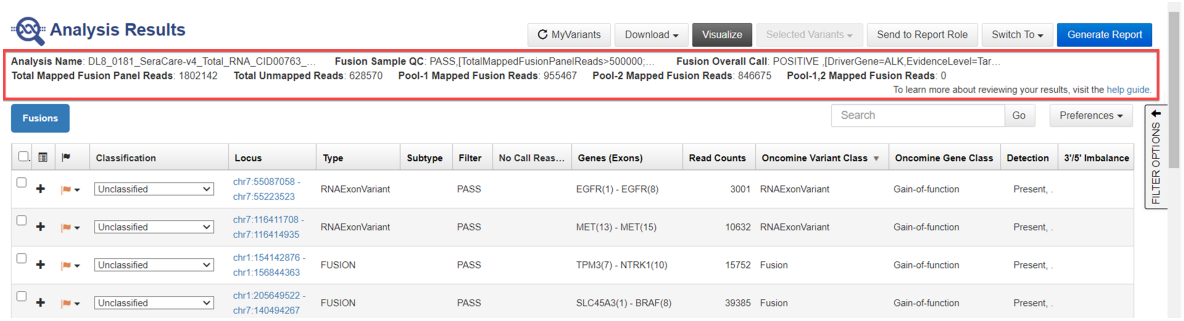
Fusion results

The **Fusions** results table lists the calls and other information for the fusions, including intragenic MET exon skipping variants, analyzed in each sample in a run.

1. Click the **Analyses** tab.
   The **Overview** screen displays a list of analyses in the **Analyses** table.

2. Click a sample result hyperlink in the **Analysis** column to open the **Analysis Results** summary screen.

3. Click the **Fusions** tab to view the **Fusions** results table.
   If using a Fusions analysis workflow, only the **Fusions** results table is displayed in the **Analysis Results** summary screen.



Column	Description
Classification	A user-defined classification selected from the list.
Locus	The chromosome positions in the reference genome that define the fusion junction.
Type	Assay type (for example, Fusion, RNA exon variant (exon skipping), RNAExon Tile, Proc Control).
Filter	Indicates the presence or absence of a fusion or RNA exon variant. When the default filter chain is applied, only the fusion/RNA exon variant calls that are designated with **PASS** are displayed in the results table. To view all calls, including calls that do not pass the required filter thresholds, apply the **No Filter** filter chain or download the **Variants (.vcf)** file. • **PASS** – indicates a high confidence call that passes all filter thresholds at a given variant position. • **FAIL** – indicates the absence of a fusion due to the variant call failing internal quality control. • **NO CALL** – while some evidence for the presence of a fusion exists, the call does not pass one or more filters that are required for a high confidence fusion call.
No Call Reason	The reason for reporting a fusion as **NOCALL** in **Filter** column.
Genes (Exon)	The name of fusion target and representative acceptor and donor exons.
Read Counts	The frequency that the fusion was detected in the sample.
Oncomine Variant Class	Variants that are known Oncomine™ annotated hotspots.
Oncomine Gene Class	Gain-of-function, or Loss-of-function
Detection	Returns: **Present**, **Absent**, **Present-Non-Targeted** or **NoCall** as suppported by the read counts dependent upon the thresholds set for detection.

(continued)

Column	Description
3'/5' Imbalance [1]	Each fusion gene exhibits a characteristic **Imbalance Score** threshold. Scores that exceed this threshold value indicate a high likelihood of the presence of the fusion in the test sample. • **Observed** = (sum of read count coverage of amplicons downstream (3') of a predicted breakpoint in a target gene) / (sum of read count coverage of all amplicons of the gene) [Read counts from test sample] • **Expected** = (sum of baseline value for amplicons downstream of the breakpoint/sum of baseline values) / (sum of baseline values of all amplicons of the gene) [Baseline values computed from normal samples]
Ratio to Wild Type	The ratio of a given variant within all wild type variants of that gene.
Norm Count Within Gene	The ratio of a given variant within all variants of that gene.
Imbalance P-value	The P-value calculated for a given variant Imbalance score.

[1] Column appears in analyses that use the exon tiling fusion detection method.

After you review, filter, and sort your Analysis Result, you can create a report (see "Generate an Analysis Results Report" on page 90), or download the analysis files (see "Download Ion Reporter™ annotation VCF or TSV files" on page 91).

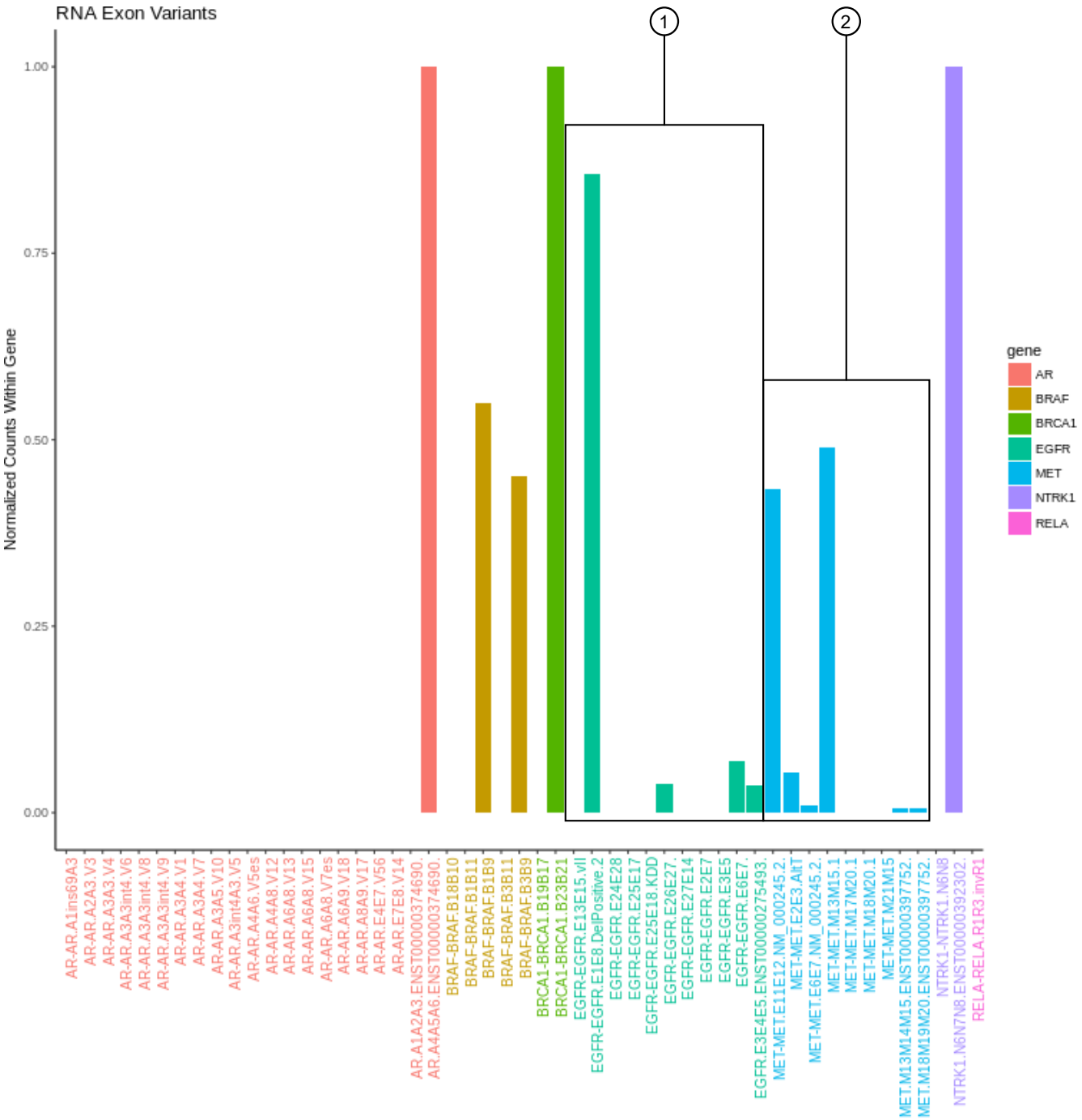
View RNA exon variants

The RNA Exon Variant data view displays a bar graph summary of intragenic exon rearrangements or fusions. The displayed RNA exon variants are defined in the BED file that is associated with an assay. The RNA Exon Variant data view is available for all RNA and Fusion assays.

1. In the menu bar, click **Analyses** ▸ **Overview**.
2. In the Analysis column, click the hyperlinked name of the analysis of interest.
3. Click the **Variants** tab, then click **Fusions**.
   The **Fusions** table opens to display fusions results.
4. In the top right corner of the screen, click **Visualization** ▸ **RNA Exon Variant** to view the **RNA Exon Variant** plot.

To return to the table view of fusions, click the browser **Back** arrow.

Representative RNA Exon Variant plots



The X-axis represents specific exon variants, where each variant is labeled with a gene ID followed by a sequence of adjacent exons. The Y-axis measures the read counts for each variant, normalized to the wild type.

① Example analysis where RNA exon 2–7 deletion occurred in the EFGR gene. The deletion of exons 2–7 resulted in an increase of normalized read counts for the EFGR variant that contains the intragenic fusion of exon 1 and exon 8 (EGFR-EGFR.E1E8.DelPositive.2), and a decrease of normalized read counts for the wild type EFGR (EFGR.E6E7).

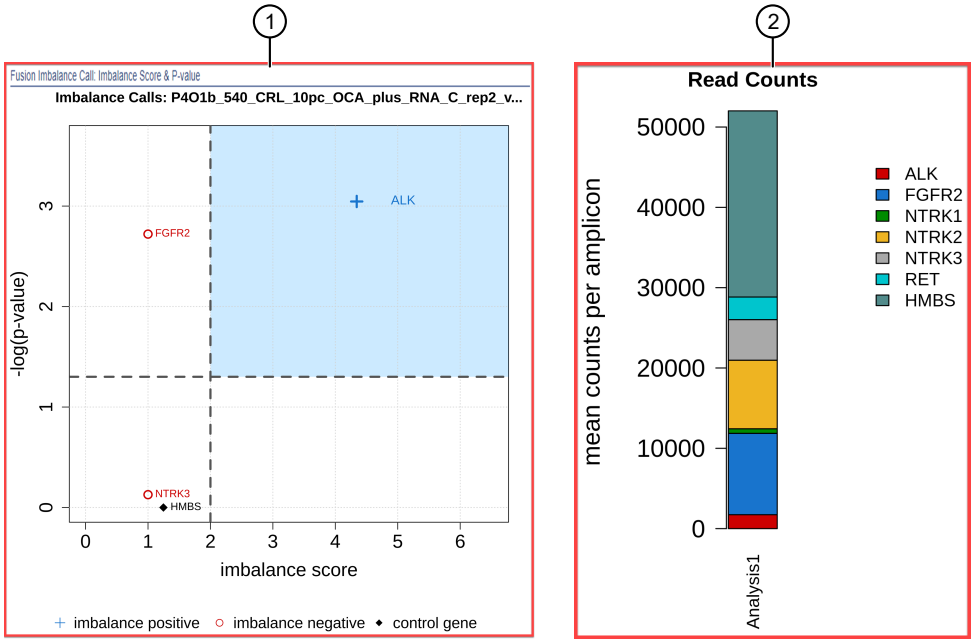
② Example analysis where exon-skipping (i.e., exon deletion) of exon 14 in the MET gene was detected. Normalized read counts for the variant representative of the exon-skip event (MET-MET.M13M15.1) are higher relative to the MET wild-type assays (for example, MET.M13M14M15.ENST00000397752.WT and MET.M17M18M19M20.ENST00000397752.WT).

View RNA Exon Tile Fusion Imbalance

The **RNA Exon Tile Fusion Imbalance** data view provides a visual representation of the RNA fusion imbalance analyses.

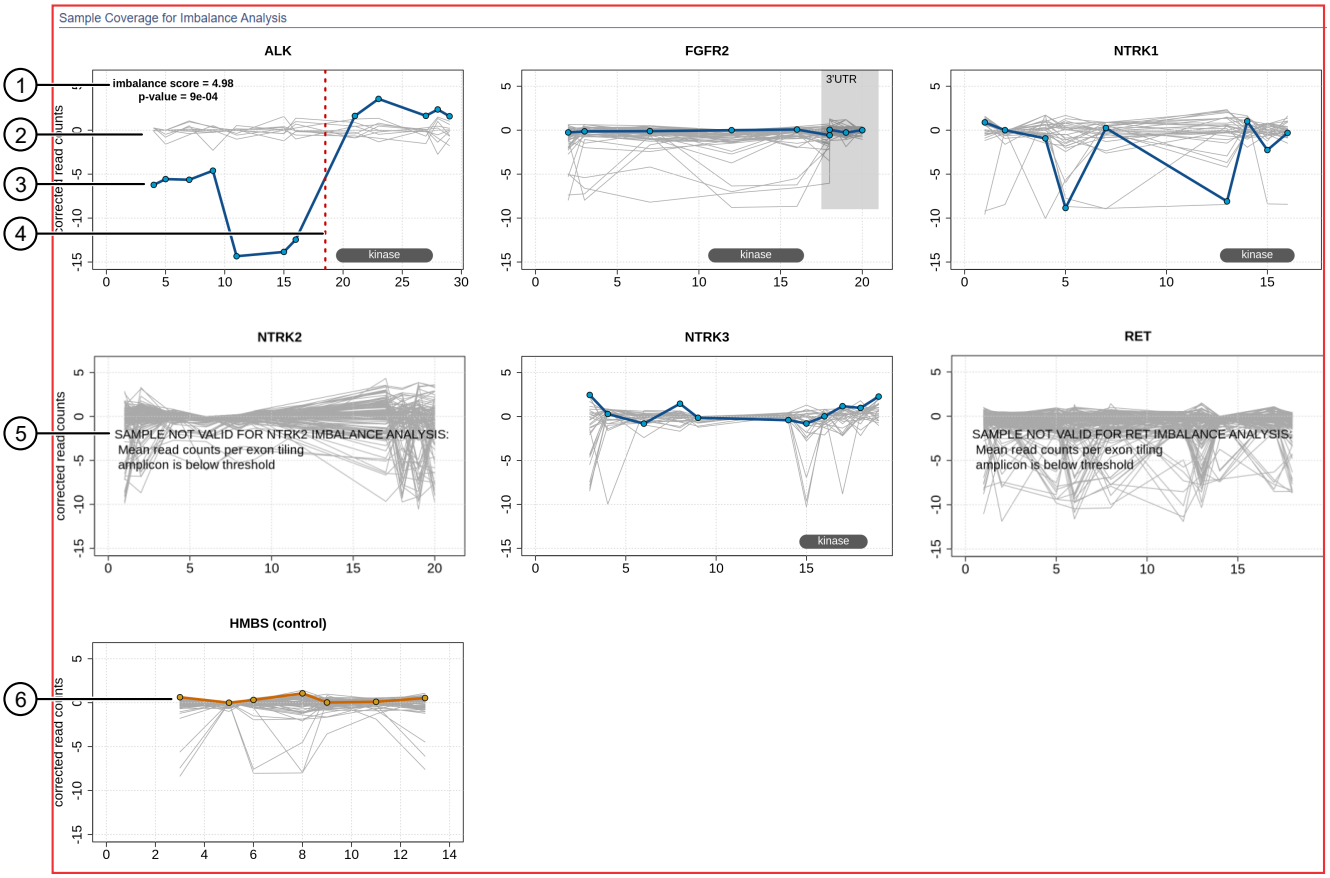
1. In the menu bar, click **Analyses** ▸ **Overview**.
2. In the **Analysis** column, click the hyperlinked name of the analysis of interest.
3. Click the **Variants** tab, then click **Fusions**.
   The **Fusions** table opens to display fusions results.
4. In the top right corner of the screen, click **Visualize**, then select the **RNA Exon Tile Fusion Imbalance** tab to view the plots.

Representative RNA Exon Tile Fusion Imbalance plots



(1) The **Fusion Imbalance Call: Imbalance Score & P-value** plot shows the imbalance scores and p-values for all the genes in the selected sample. The dashed gray lines mark the threshold for an imbalance call, which is applied to all genes across all samples. Points that fall within the blue shaded area of the plot represent fusion-positive genes (+). All other points that are outside of the blue shaded area represent fusion-negative genes (○). Control genes are marked with ◆.

(2) The **Normalized Read Depth by Gene** plot shows the mean read counts of each gene that is captured on the chip for the selected sample. For each gene, the read counts are normalized to the number of amplicons.



(1) The **Sample Coverage for Imbalance Analysis** plots show the expression profile for each exon-exon tiling amplicon for each gene. The Y-axis represents the normalized coverage after baseline correction. The X-axis represents individual exon-exon junctions, which are listed from 5′ to 3′. The **imbalance score** and **p-value** are listed in the panel of each gene that was called positive for fusion.

(2) Baseline (a cluster of gray lines), generated from a fusion-negative sample.

(3) Test sample corrected read coverage (blue line), normalized to the baseline. Each point on the line represents a unique exon-exon junction that was covered by the assay and normalized to the baseline.

(4) Predicted range for the fusion break point for a fusion-positive gene (dashed red line).

(5) If the collected data are insufficient to determine an imbalance score, the **SAMPLE NOT VALID FOR <gene> IMBALANCE ANALYSIS** message appears in the panel for that gene.

(6) Sample coverage profile for the control gene (orange line).

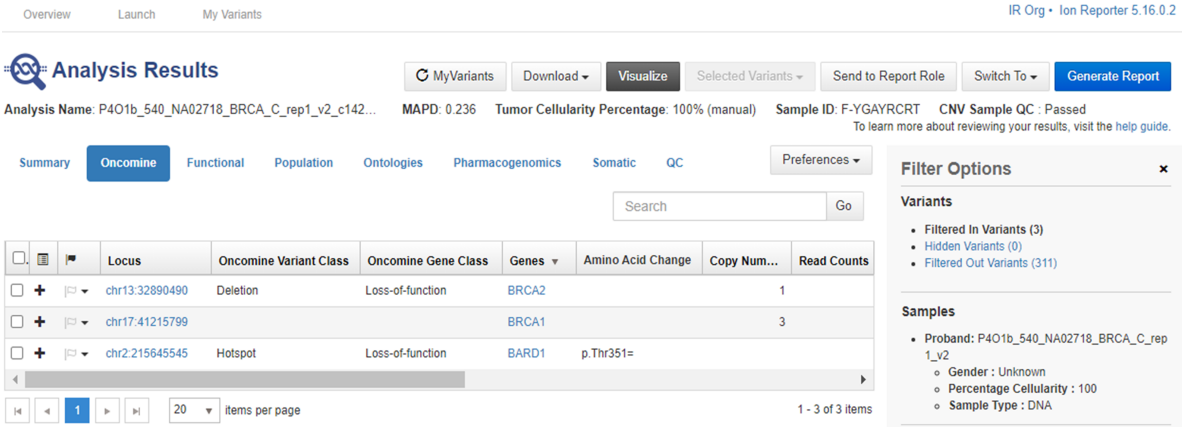
To return to the table view of fusions, click the browser **Back** arrow.

Oncomine™ BRCA analysis results

View Oncomine™ BRCA analysis results

The provided Ion Reporter™ Software 5.16 or later Oncomine™ BRCA Expanded analysis workflows and any panel-specific analysis workflows created by copy/editing the Oncomine™ BRCA Expanded analysis workflow now include end-to-end coverage of both BRCA1 and BRCA2 genes with optimized variant calling for SNV, long deletions, exon deletion and duplication, and whole somatic gene deletion variants in somatic and germline samples with high sensitivity.

1. In the **Analyses** tab, click **Overview**.
2. Search, sort, or filter the **Analyses** table to find the sample result of interest.
3. In the **Analysis** column, click the hyperlink for the sample result of interest to open the **Analysis Results** screen for your BRCA analysis.



4. The following data for the BRCA results is available in the **Analysis Results** screen:
   - A summary of called variants and their genotypic and functional properties, and sort and select variants of interest. Variants that are listed include SNVs, InDels, germline long exon deletions, duplications, and germline and somatic whole gene deletions and duplications.
   - Metrics and information at the top of the screen.

Item	Description
Analysis Name	The name of the analysis.
Cancer Type	The type of cancer, as defined by the sample attribute in Torrent Suite™ Software, if sample data is transferred by Torrent Suite™ Software.
MAPD	The MAPD (Median of the Absolute values of all Pairwise Differences) metric is an estimate of coverage variability between adjacent amplicons. The default threshold is 0.65. As a result, sample results with a MAPD above this value should be viewed with lower confidence.

(continued)

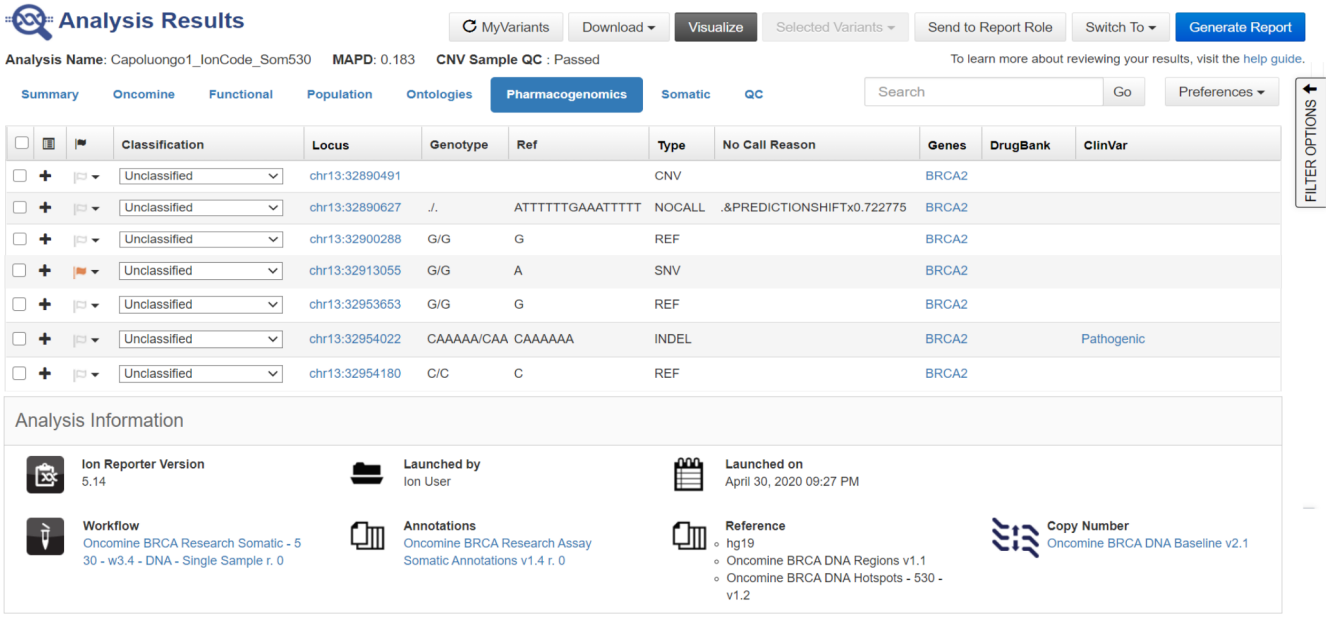
Item	Description
CNV Sample QC	A quality control score that is based on the MAPD threshold. **CNV Sample QC** is PASSED or FAILED. If failed, a reason is provided in a message.
Sample ID	**Note:** If the sample data is transferred from Torrent Suite™ Software and the SampleID plugin was run, a Sample ID is displayed at the top of the **Analysis Results** screen, and on the **Generate Report** page in the **Sample Information** section.

- Click **Summary** to view a summary of the called variants. Select a classification from the dropdown list to assign a classification to a variant. The following variant types are available for a BRCA analysis.

Type	CNV Subtype	Description
CNV	BigDel	Deletion of at least one exon
	BigDup	Duplication of at least one exon
	GeneCNV	Whole BRCA1/BRCA2 gene deletion or duplication
	LongDel	A specific 40 bp deletion in BRCA1
	NOCALL	Read count differs from baseline by non-integer amount; evidence for a BigDel or BigDup call is weak
	REF	Read count matches reference baseline
SNV	—	Single nucleotide substitution
MNP	—	Multiple nucleotide polymorphism at adjacent nucleotide positions
INDEL	—	Single or multiple nucleotide insertion or deletion

- Click **Functional** to view other functional annotations and use the annotations to classify, sort, and filter variants.

- Click **Pharmacogenomics** to view the **ClinVar** column. Click the link in the **ClinVar** column for a selected variant to open an NCBI ClinVar website where information about the ClinVar variant annotation is available.



Visualize the BRCA report

Core or custom Oncomine™ BRCA Expanded panels used with Ion Reporter™ Software enables the detection and visualization of whole exon and multiple exon deletion in BRCA1 and BRCA2 genes in somatic and germline samples with high sensitivity.

1. In the **Analysis Results** screen, click **Visualize**.

   When you open a visualization for Oncomine™ BRCA Expanded analysis results, you can view a **BRCA Report** that shows a boxplot of read counts of each BRCA1 and BRCA2 exon that are normalized to the Oncomine BRCA DNA Baseline. The Post-Corrected view is displayed by default. Click the Pre-Corrected tab to view the uncorrected raw data.

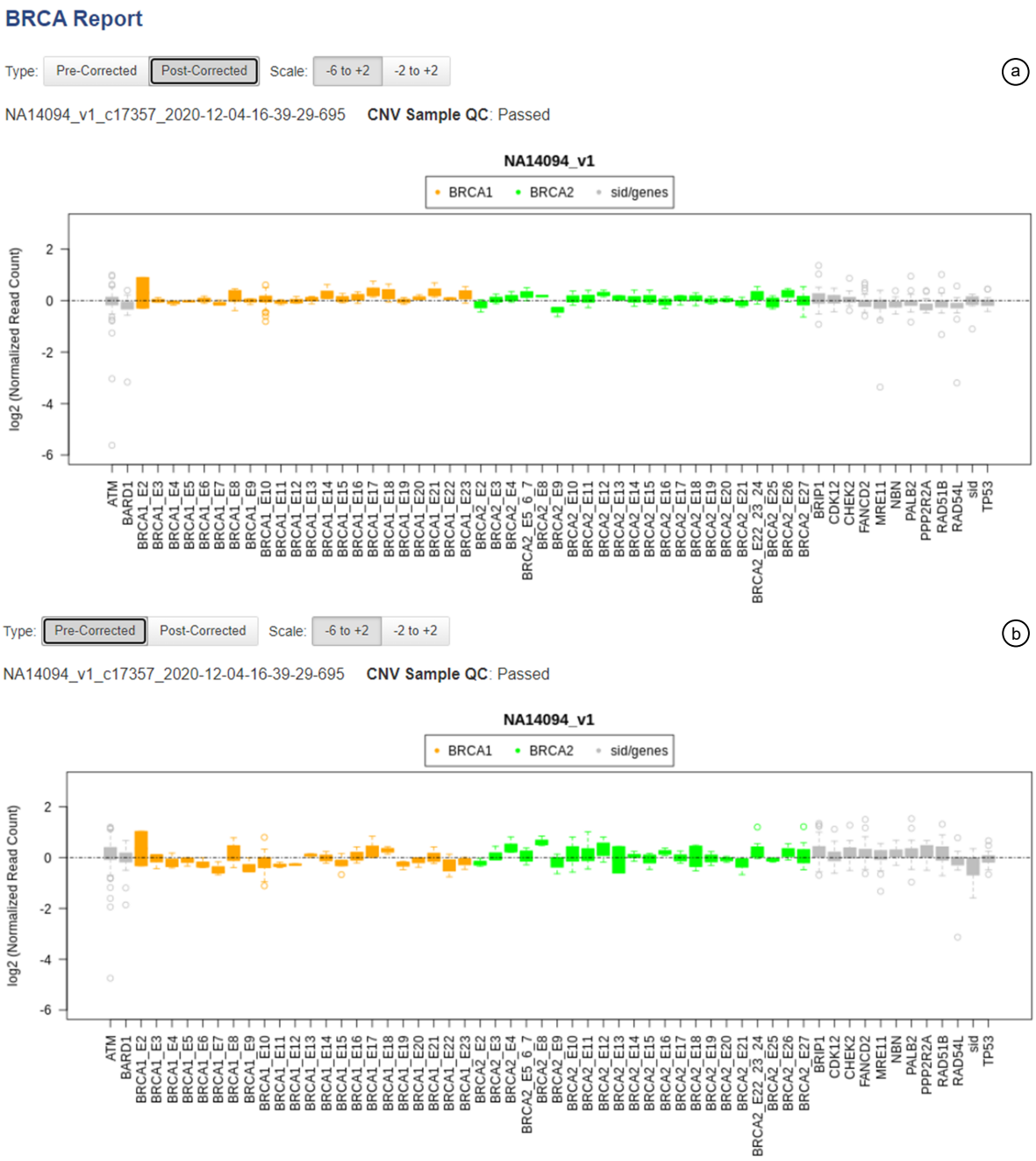
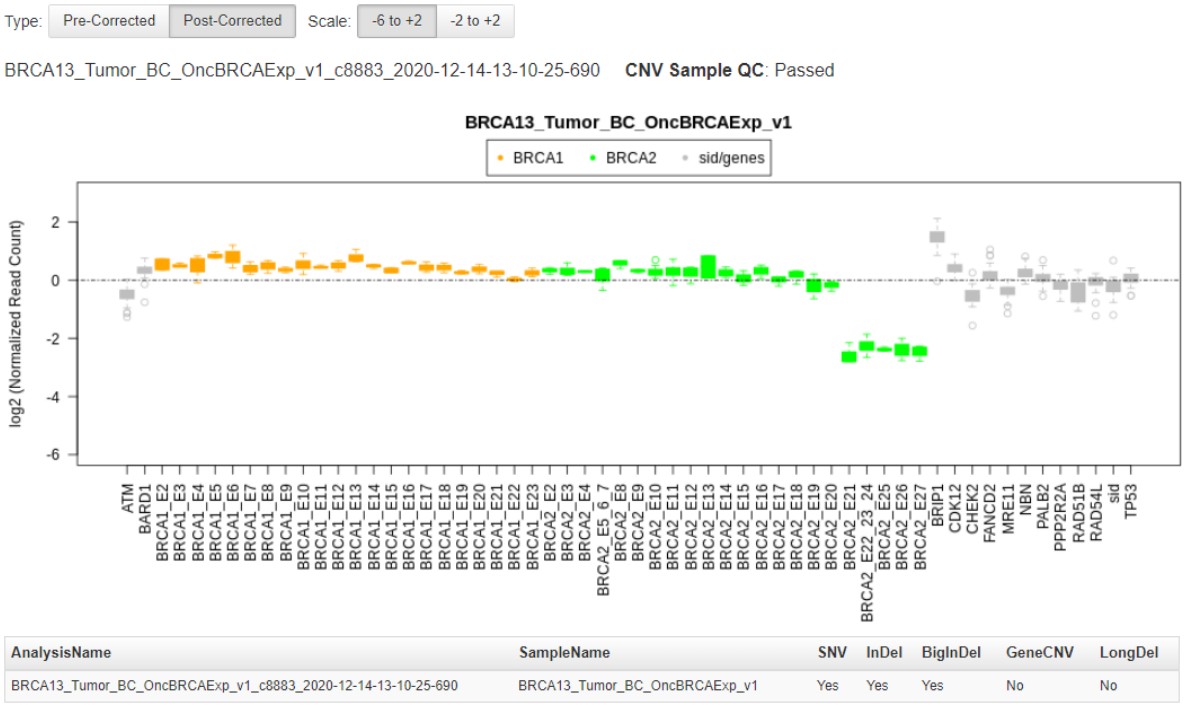


Figure 2 Normal sample, no BRCA whole-gene or exon deletion

Example post-correction boxplot (Figure 2 **a**) of a tissue normal sample without whole-gene or exon deletions in either the BRCA1 or BRCA2 genes. Notice the data have less variability relative to the pre-corrected view (Figure 2 **b**).

In Ion Reporter™ Software 5.16, the Post-Corrected view normalizes the abundance of the amplicons in BRCA1 and BRCA2, bringing the median coverage of amplicons in BRCA1 and BRCA2 to comparable values. This correction is used to detect exon deletions in the BRCA1 or BRCA2 genes (Figure 3). As a result the Post-Corrected view does not show any whole-gene deletions or duplications in BRCA1 or BRCA2 (for example, somatic CNVs). This behavior is different than in the previous version of Ion Reporter™ Software (5.14), where whole-gene deletions

were visible in the Post-Corrected view. To view whole-gene deletions or duplications, look at the Pre-Corrected tab (Figure 4) or IRGV.



AnalysisName	SampleName	SNV	InDel	BigInDel	GeneCNV	LongDel
BRCA13_Tumor_BC_OncBRCAExp_v1_c8883_2020-12-14-13-10-25-690	BRCA13_Tumor_BC_OncBRCAExp_v1	Yes	Yes	Yes	No	No

Figure 3 Tumor sample with BRCA exon deletion

Example Post-Corrected boxplot of a tumor sample with an exon deletion in the BRCA2 gene.

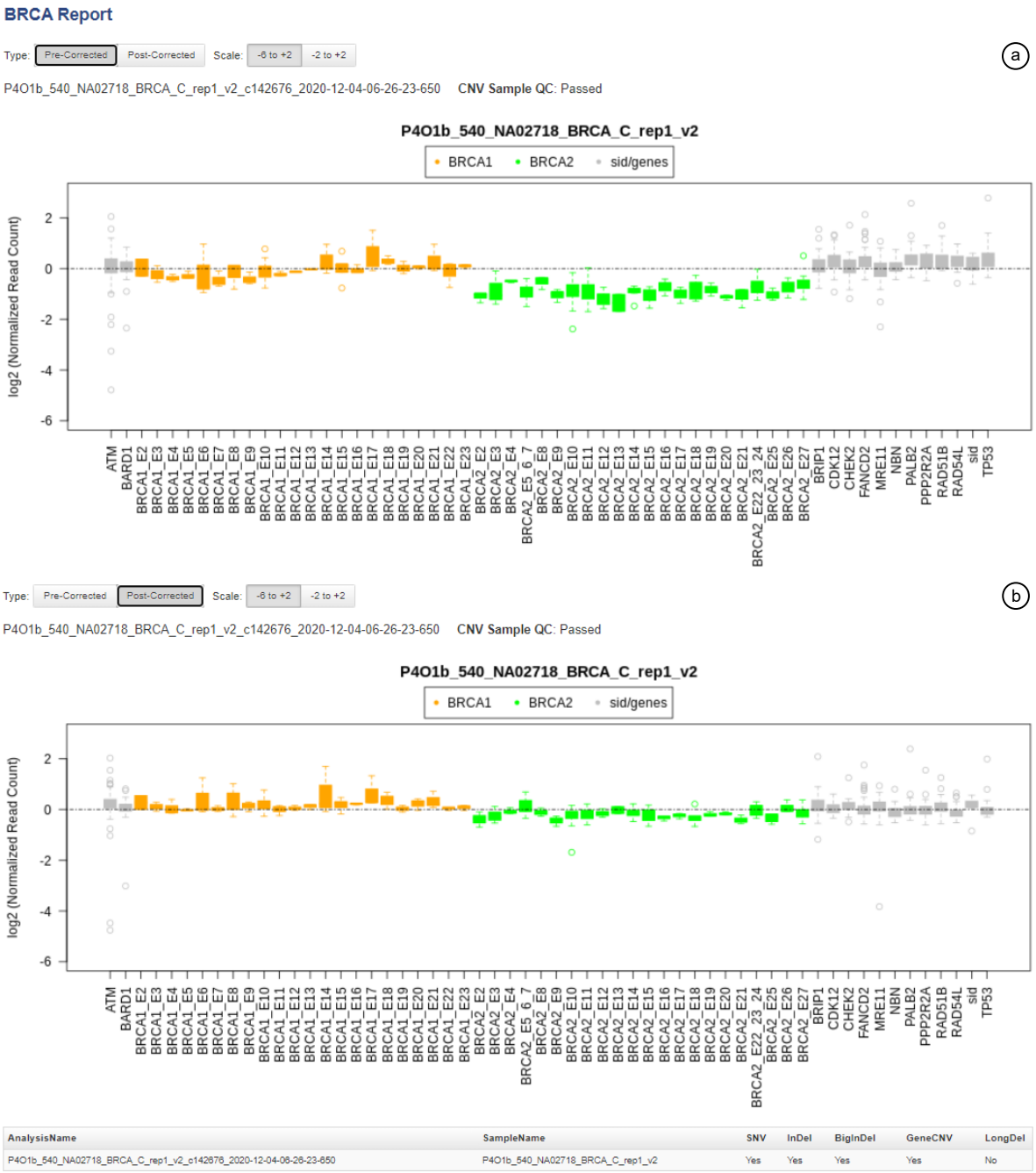


Figure 4 Tumor sample with BRCA2 whole-gene deletion

Example Pre-Corrected boxplot (Figure 4 **a**) of a tumor sample with a whole-gene deletion in the BRCA2 gene. Notice that the whole-gene deletion is not visible in the Post-Corrected view (Figure 4 **b**).

Note: If either of the BRCA1 or BRCA2 genes is amplified, it is possible that the other BRCA gene may be incorrectly described as being deleted. To identify whether a BRCA gene has been amplified or the other BRCA gene deleted, review the Pre-Corrected boxplot to compare the abundance of the amplicons in both the BRCA1 and BRCA2 genes compared to all the other genes.

---

2. Click the **IRGV** tab to view exon deletions or duplications on chromosomes 13 and 17.

① Click to open the **Sample Analysis Summary** in a separate browser window

② Zoom in and zoom out for the selected chromosome

③ Click to select and view a chromosome

④ Slide the **MAPD filter** to adjust the threshold value. This filter applies to the entire sample. If the reported MAPD value is above the selected **MAPD filter** value, no CNVs are displayed in the BRCA Report.

⑤ Scroll down in this section to see more of the alignments or hotspots.

⑥ Oncomine BRCA Expanded designed BED file alignment. This **IRGV** track shows the alignment of the amplicons to the reference sequence.

3. Click **IRGV Export & Preferences** to open the visualization in IGV, export the whole genome view or the IRGV tracks to a PNG file, or show IRGV preferences.

Generate an Analysis Results Report

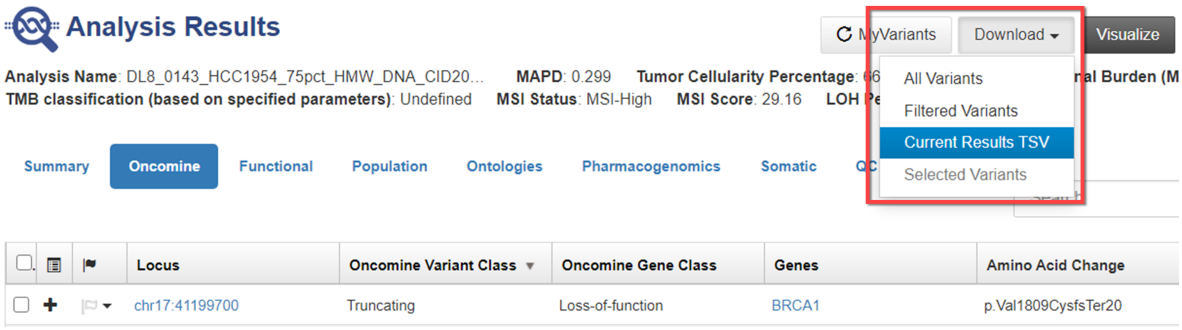
After you have reviewed, filtered, and sorted your Analysis Result, you can download an Analysis Report. The procedure described here includes creating and formatting a report template.

1. In the Analysis Results screen for your sample, click **Generate Report**.
   The **Generate Report** workflow bar opens to the **Configuration** step. The sections of the report can be rearranged, deleted, or edited.
2. Hover over the various sections and icons to view instructional text to help you format your report output.
3. Enter information in editable fields (for example, edit the report name or enter background information).
4. *(Optional)* Click **Save As New Template** to save your reconfigured report template for future use with other sample results.
5. Click **Next**, a live preview of your report is displayed.
6. Click **Lock & Publish** to generate the final Analysis Report.
7. Click **Download**.

Download Ion Reporter™ annotation VCF or TSV files

Variant call format (VCF), and tab separated values (TSV) files of the complete or filtered results can be downloaded from the **Analysis Results** screen.

1. Click **Download**, then select **All Variants**, **Filtered Variants**, **Current Results TSV**, or **Selected Variants**.

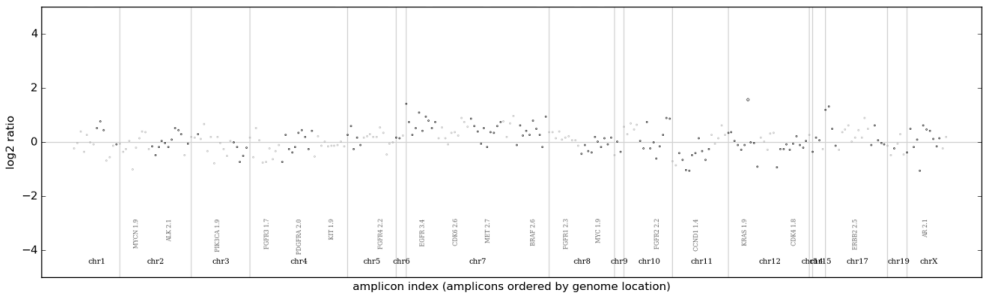


   - All variants—A VCF file that contains all variants that are included in the analysis.
   - Filtered variants—A VCF file that contains the variants which were filtered IN for the analysis.
   - Selected variants—A VCF file that contains your selected variants from the analysis. Each variant to be included in the selected variants VCF file must be selected in the Analysis Results before the files are downloaded.
   - Current Results TSV—A tab separated values (TSV) file of the analysis results of the current analysis.

2. Click **Home** ▸ **Notifications** to open the **Notifications** screen, then click (Download) next to the file name to download your results.

   Alternatively, select one or more rows, then click **Download**.

   The software generates a compressed directory named <analysis name>_All.zip that is downloaded. Extract the contents of the ZIP file to access the following 4 folders: CNV_VCIB, QC, Variants, Results, and Workflow_Settings. Within the Variants folder, you'll find the Oncomine™ annotated VCF file.

Folder	Contents of folder
CNV_VCIB	Contains an image file (`cn_results.png`) of the copy number determination for all amplicons. 
QC	Contains a PDF of the QC report, and a folder containing coverage statistics files.

(continued)

Folder	Contents of folder
Variants	Contains a folder with the following files: • Intermediate and Oncomine™-annotated .VCF files • TSV files that contain Oncomine™-filtered and all somatic variants
Workflow_Settings	Contains folders with the following files: • A text file that describes settings used for the analysis. Open the file with a text editor. • Configuration files used by the Ion Reporter™ Software in the analysis workflow settings.

You can view the extracted files individually, or upload a VCF files to an ancillary software application such as the Ion Torrent™ Oncomine™ Reporter for further analysis.

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 alternating 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 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 and carefully aliquot into 96-well plates.
- When using a Qubit™ Fluorometer or the Agilent™ 2100 Bioanalyzer™ 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.
- If the unamplified library yield is below 50 pM, libraries can be rescued with library amplification. Combine 25 µL of unamplified library with 71 µL of 1X Library Amp Mix and 4 µL of 25X Library Amp Primers. Seal the plate and perform 5–10 library amplification cycles as follows:

Stage	Temperature	Time
Hold	98°C	2 minutes
5–10 cycles	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold (up to 1 hour)

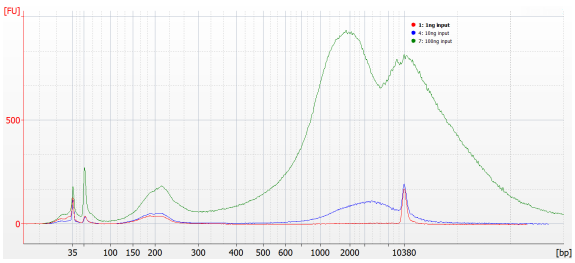
- When amplifying multiple samples in a single PCR plate, ensure that the input across the samples is equivalent so that the selected cycle number is optimal for all the samples in the run.
- If you combine aliquots of captured libraries before adding Equalizer™ Beads, save the unused portions of amplified 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.

Troubleshooting

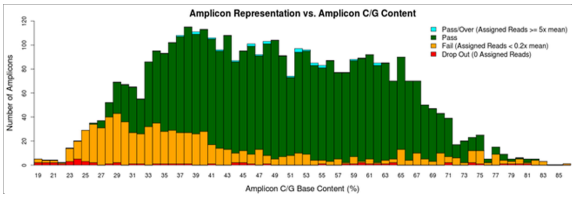
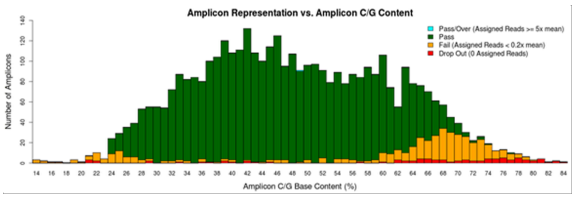
Library yield and quantification

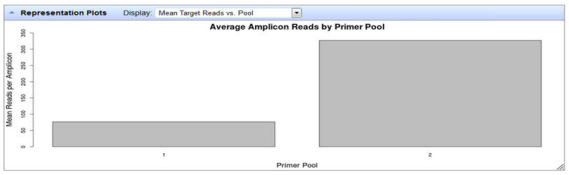
Observation	Possible cause	Recommended action
Library concentration is low—general **Details:** (Library concentration is NOT indicative of quality.)	Sample DNA or RNA was mis-quantified.	Requantify sample DNA using the TaqMan™ RNase P Detection Reagents Kit; quantify RNA with a Qubit™ Fluorometer.
	Residual ethanol in sample DNA or RNA inhibited target amplification.	Incubate 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.

Observation	Possible cause	Recommended action
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.
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.

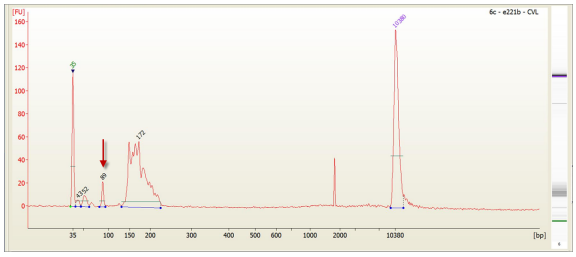
Low amplicon uniformity (DNA only)

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).
	Digested amplicons were denatured.	Use the 60°C/20 minute temperature incubation during the amplicon digestion step.
Long amplicons are under-represented (short library reads)	Sample DNA or RNA was degraded.	Use an FFPE assay design for degraded or low quality samples.
	PCR was inefficient.	Double the anneal and extend time.
	Too few nucleotide flows were used.	Use an appropriate number of flows to sequence through amplicons. **Note:** Use 550 flows as a default setting when sequencing libraries prepared from most Ion AmpliSeq™ On-Demand Panels. In rare instances, amplicons can be longer than 325 bp and can require 650 flows to achieve end-to-end reads, if needed. Determine amplicon length from the panel BED file. Use the templating and sequencing paramaters for 200 bp.

Observation	Possible cause	Recommended action
Long amplicons are under-represented (short library reads) *(continued)*	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.
	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.

Observation	Possible cause	Recommended action
Pool representation is not balanced **Details: 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.

Observation	Possible cause	Recommended action
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.
	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 information

■ Configure the IonReporterUploader plugin in Torrent Suite™ Software 101

Configure the IonReporterUploader plugin in Torrent Suite™ Software

1. Sign in to the Torrent Suite™ Software.
2. Click ⚙ ▾ **(Settings)** ▸ **Ion Reporter Configure**.
3. In the **Ion Reporter Uploader Account Configuration** screen, click ＋ **Add Account** ▸ **Ion Reporter**.
4. In the **Add Ion Reporter account** screen, enter the following information into the fields:

Field	Directions
Server Type	Select:[1]
Display Name	Enter a meaningful name of your choice. This name is used in the run plan template wizard and is seen by other Torrent Suite™ Software users. Use only alphanumeric characters, spaces, and underscores.
Server	Enter:[1]
Port	Enter: 443
Username	Enter your Ion Reporter™ Software username (your email address)
Password	Enter your Ion Reporter™ Software password

[1] Ask your Ion Reporter™ Server administrator for these values.

5. The "Default Account" is the account that is configured by default in run templates and run plans. If this account is the main account to be used for file transfers, enable the **Default Account** checkbox.

   Note: You can always change this selection in the Planned Run template workflow bar and in the Upload to IR quick link.

6. Click **Get Versions**, select **Ion Reporter 5.16 or later**, then click ✓**Add**.

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

- Amplify the library 102
- Purify the amplified library 103
- Qubit™ Fluorometer: Quantify the library and calculate the dilution factor 105
- Agilent™ 2100 Bioanalyzer™ instrument: Quantify the library and calculate the dilution factor . 106
- Store libraries 106

IMPORTANT! Quantification of Oncomine™ tumor specific assay libraries using the Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ has been demonstrated but not validated. We recommend using the Ion Library TaqMan™ Quantitation Kit to quantify unamplified libraries.

Before quantification with a Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ instrument, Oncomine™ tumor specific panel libraries must be amplified to enrich amplifiable material and obtain sufficient material for accurate quantification. Amplify the library using 1X Library Amp Mix, then purify. Quantify the library using a Qubit™ Fluorometer or the Agilent™ 2100 Bioanalyzer™ instrument. Amplified libraries typically have yields of 2,000–10,000 pM. Yield is not indicative of library quality. After quantification, determine the dilution factor that results in a concentration of ~100 pM, which is appropriate for template preparation using an Ion template kit.

Amplify the library

1. Remove the plate with purified libraries from the plate magnet, then **add 50 µL of 1X Library Amp Mix** and **2 µL of 25X Library Amp Primers** to each bead pellet.

   Note: The 1X Library Amp Mix and 25X Library Amp Primers 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:
    - or
    - “Agilent™ 2100 Bioanalyzer™ instrument: Quantify the library and calculate the dilution factor” on page 106.

    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.

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.

D

Equalize the library

- Before you begin 107
- Amplify the library 108
- Wash the Equalizer™ Beads (if not previously performed) 109
- Add Equalizer™ Capture to the amplified library 109
- (Optional) Combine captured libraries 109
- Add Equalizer™ Beads and wash 110
- Elute the Equalized library 111
- Store libraries 111

IMPORTANT!

- Use of the Ion Library Equalizer™ Kit in preparation of Oncomine™ tumor specific assay libraries has been demonstrated but not validated.
- We recommend starting with the qPCR library quantification method (see "Quantify the library by qPCR and calculate the dilution factor" on page 50). 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 DNA or RNA from FFPE tissue), 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 Oncomine™ tumor specific panel 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 1X Library Amp Mix** (black cap) and **2 µL of Equalizer™ Primers** (pink cap), or 2 µL of 25X Library Amp Primers, to each bead pellet. The 1X Library Amp Mix and primers can be combined before addition.

2. Seal the plate with a clear adhesive film, vortex thoroughly, then centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

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

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

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

5. (Optional) If possible after thermal cycling, 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.

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.

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.

Oncomine™ tumor specific Sample ID Panel

Using the Ion AmpliSeq™ Sample ID Panel

Each Oncomine™ tumor specific panel includes the Ion AmpliSeq™ Sample ID primers to allowing you to generate unique sample signatures for each sample. To generate an Ion AmpliSeq™ Sample ID signature, you must include the Ion AmpliSeq™ Sample ID Panel in your Planned Run.

Note:

- Do not add additional Ion AmpliSeq™ Sample ID primers to your library preparation reactions.
- The Ion AmpliSeq™ Sample ID Panel 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.

---

1. Sign in to the Torrent Suite™ Software.
2. When creating a Planned Run using the Planned Run wizard. In the **Kits** step, in the **Control Sequence** dropdown list, select **Ion AmpliSeq Sample ID panel**.
3. In the **Plugins** step
   a. Select the **coverageAnalysis** plugin checkbox, then click **Configure** (see “coverageAnalysis plugin” on page 114).
   b. 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.

   c. Select the **sampleID plugin**. A Sample ID Report is then automatically generated after the run.
4. Following sequencing, select the **Data** tab in the Torrent Suite™ Software, then click **Completed Runs and Results**.
5. In the list of runs, find the run of interest, then click the link in the **Report Name** column.
6. In the left navigation menu, click **sampleID** to view the plugin summary.
7. Click **sampleID.html** to open the Sample ID Report.

8. Click the link in the **Barcode Name** column to open a sample specific Sample ID Report.

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

Review sequencing results

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 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**.

(continued)

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.
Minimum 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.
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.

(continued)

Setting	Description
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.
Non-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 summary lists the barcodes, the samples, the number of mapped reads, the percentage of on target reads, mean base coverage depth, and base coverage uniformity. 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.

For more information on how to review and interpret reads statistics and output files, see .

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 coverageAnalysis summary pane.
4. In the coverageAnalysis barcode summary, in the **Barcode Name** column, click a link to open a detailed **Coverage Analysis Report** for that barcoded sample.
   Alternatively, click the **coverageAnalysis.html** link to open the summary table for all barcodes in a new window.
5. In the **Coverage Analysis Report**, review the plugin results. 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. The following tables describe the statistics that are generated by the coverageAnalysis plugin. The statistics that are displayed in your report depend on the type of library that is used in your sequencing experiment. 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.

General statistics

Statistic	Description
Number of mapped reads	The total number of reads mapped to the reference genome.
Percent reads on target	The percentage of filtered reads mapped to any targeted region relative to all reads mapped to the reference. If no target regions file is specified, this value will be the percentage of reads passing uniquely mapped and/or non-duplicate filters, or 100% if no filters were specified. A read is considered on target if at least one aligned base overlaps at least one 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.

Amplicon read coverage statistics

The following statistics describe the reads that are assigned to specific amplicons. Each sequence read is assigned to exactly one of the amplicons specified by the targets file. If a read spans multiple amplicon targets, the target region that the reads covers most is assigned. In the event of a tie, the target that is the closest to the 3' end is assigned.

Statistic	Description
Number of amplicons	The number of amplicons that is specified in the target regions file.
Percent assigned amplicon reads	The percentage of reads that were assigned to individual amplicons relative to all reads mapped to the reference. 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, 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 amplicons that had at least 20% of the average number of reads per amplicon. Cumulative coverage is linearly interpolated between nearest integer read depth counts.
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 ≥10 reads and the percentage of forward or reverse reads to total reads is greater than 70%. Amplicons with <10 reads are considered to have no strand bias.

Amplicon read coverage statistics *(continued)*

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 base composition bias	A number that represents the proportion of amplicons showing low representation (<0.2x mean reads) in the lower and/or upper quartiles of amplicons ordered by increasing G/C 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. An RMS (root mean square) value is used so that a bias greater in either upper or lower quartiles produces a larger value than a mean bias seen more equally in both outer quartiles. The value is 0 if the uniformity of amplicon coverage metric is 100%, however, the value is not necessarily high at lower amplicon uniformity.

Target base coverage statistics

The following statistics describe the targeted base reads of the reference. A base covered by multiple target regions is counted only once per sequencing read.

Statistic	Description
Bases in target regions	The total number of bases in all specified target regions of the reference.
Percent base reads on target	The percent of all bases covered by reads aligned to the reference that covered bases in target regions. Clipped bases, deletions, and insertions (relative to the reference) are not included in this percentage. If no specific target regions were specified, the whole genome is the targeted regions.
Average base coverage depth	The average number of reads of all targeted reference bases. This is the total number of base reads on target divided by the number of targeted bases, and therefore includes any bases that had no coverage.
Uniformity of base coverage	The percentage of bases in all targeted regions (or whole genome) that is covered by at least 20% of the average base coverage depth reads. Cumulative coverage is linearly interpolated between nearest integer base read depths.
Target base coverage at *N*x	The percentage of target bases covered by at least *N* reads.
Target bases with no strand bias	The percentage of all target bases that did not show a bias toward forward or reverse strand read alignments. An individual target base is considered to have read bias if it has ≥10 reads and the percentage of forward or reverse reads to total reads is greater than 70%. Target bases with <10 reads are considered to have no strand bias.
Percent end-to-end reads	The percentage of on-target reads that fully cover their assigned amplicon (insert) 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.

Example Coverage Analysis Report

The detailed Coverage Analysis Report that is available when you select a barcode in the coverageAnalysis plugin summary for a run includes a variety of reads statistics.

Coverage Analysis Report

Sample Name: P4O1b_540_H16251T_Prostate_C_rep1

Library type: AmpliSeq DNA and Fusions
Reference: hg19 (DNA)
Target regions: P4O_Val_Oncomine_Prostate.20191120_designed
Read filters: Sample tracking

Number of mapped reads	1,865,419
Percent reads on target	94.52%
Percent sample tracking reads	0.76%
Average base coverage depth	2,175
Uniformity of base coverage	92.87%

Amplicon Read Coverage		Target Base Coverage	
Number of amplicons	937	Bases in target regions	93,872
Percent assigned amplicon reads	94.52%	Percent base reads on target	93.69%
Average reads per amplicon	1,882	Average base coverage depth	2,175
Uniformity of amplicon coverage	93.60%	Uniformity of base coverage	92.87%
Amplicons with at least 1 read	99.79%	Target base coverage at 1x	99.88%
Amplicons with at least 20 reads	99.15%	Target base coverage at 20x	99.18%
Amplicons with at least 100 reads	98.29%	Target base coverage at 100x	98.48%
Amplicons with at least 500 reads	90.29%	Target base coverage at 500x	90.78%
Amplicons with no strand bias	98.08%	Target bases with no strand bias	98.06%
Amplicons reading end-to-end	94.56%	Percent end-to-end reads	91.48%
Amplicon base composition bias	0.286		

Example charts generated by the coverageAnalysis plugin

Many of the charts in the detailed **Coverage Analysis Report** include a **Plot** menu that allows you to change characteristics of the chart. For example, you can show both strands (see callout 3 in Figure 9).

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

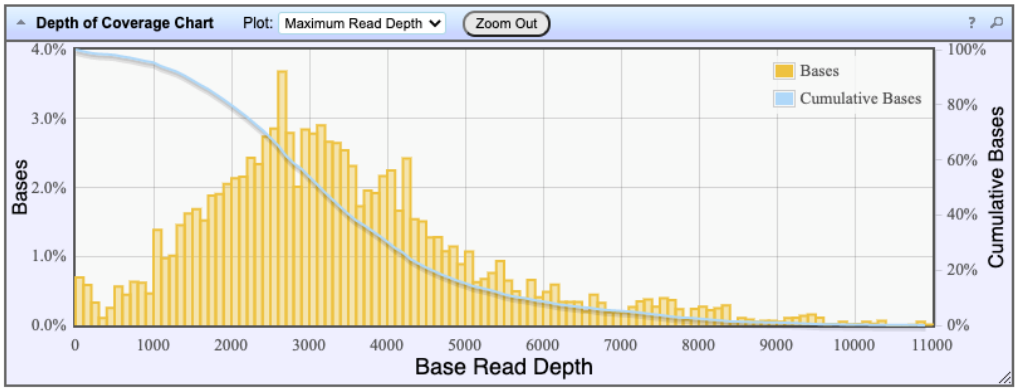


Figure 5 Representative Depth of Coverage Chart

The Depth of Coverage Chart shows the distribution of targeted base coverage. The X-axis represents the base read depth. The left Y-axis represents the number of reads at a given base read depth or a range (bin) of base read depths, as a percentage of the total number of base reads. The right Y-axis represents the cumulative count of the number of reads at a given read depth or greater, as a percentage of the total number of reads. The individual orange bars represent the percentage of reads in the specific range of base read depths. The blue curve measures the cumulative reads at a given base read depth or greater. 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 can click a data point to open a detail pane for that data. For example, in the Depth of Coverage Chart in Figure 5, click an individual orange bar to open the detail pane for bases within a specific range of base read depths. Click a point on the blue curve to open the detail pane for cumulative bases at that base read depth or greater. For example detail panes, see Figure 6. Similarly, you can click any point or bar within the Amplicon Coverage chart to view details for each amplicon coverage bin (see Figure 8).

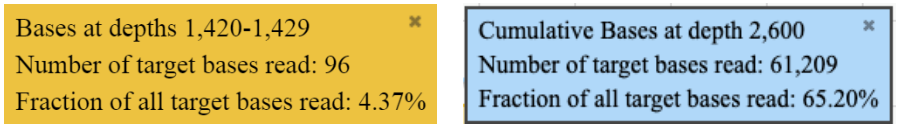


Figure 6 Example detail panes

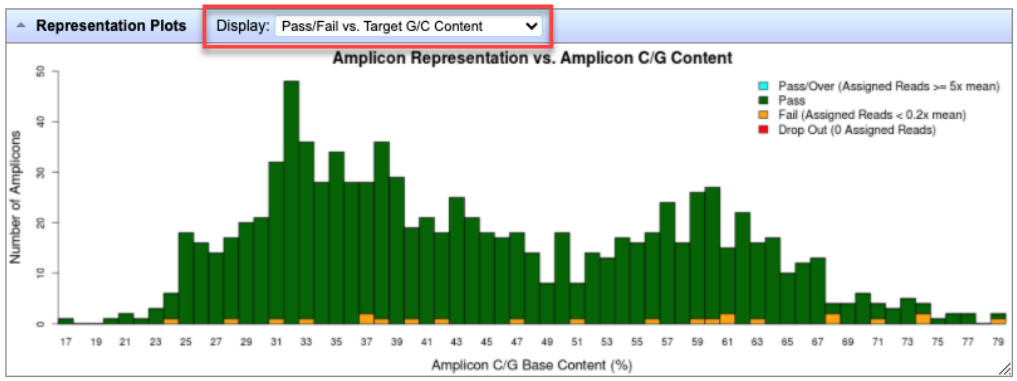


Figure 7 Representation Plots

Use the **Display** list to view different amplicon representation plots. This figure shows an example Pass/Fail vs. Target G/C Content plot.

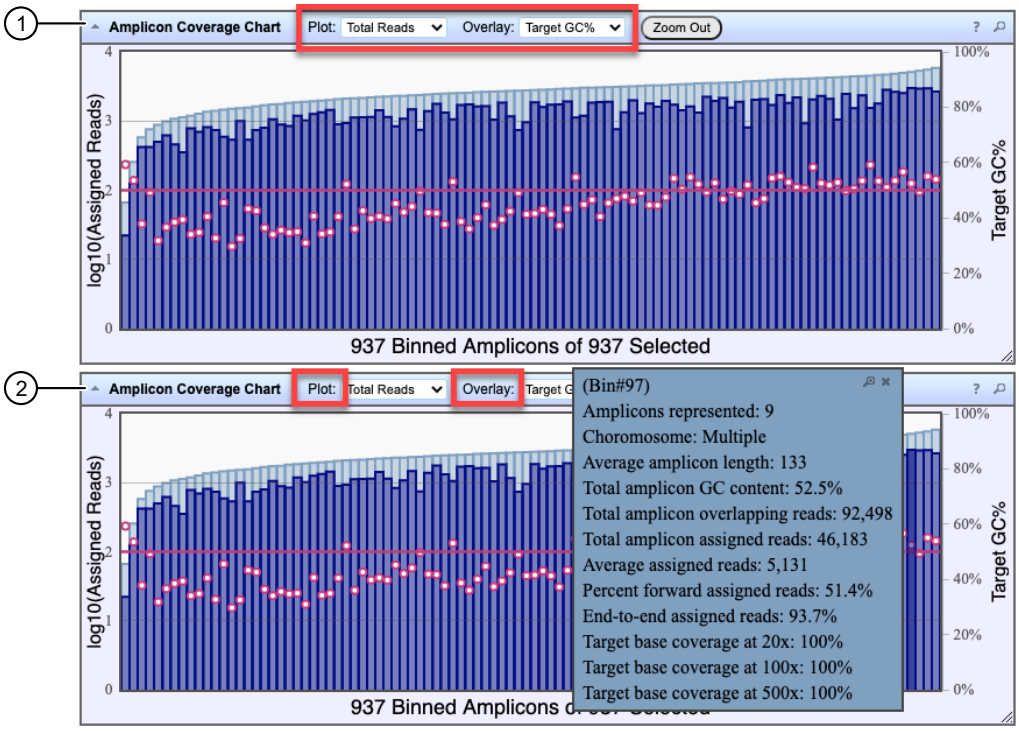


Figure 8 Representative Amplicon Coverage Chart

The Amplicon Coverage charts summarize the amplicon coverage results. Several plots views are available. The X-axis in all plots represents individual amplicons. The left Y-axis in all plots represents assigned reads (on a base 10 log scale). Use the **Plot** and **Overlay** lists to customize the chart view. The right Y-axis varies depending on the plot view that is selected from the **Overlay** dropdown list. Click on any bar or any point on the chart to view the detail pane for that data.

(1) Example Total Reads plot with a Target GC percentage overlay

(2) Click on a bar to open the detail pane for the specific amplicon. Click **View in Reference Coverage Chart** to zoom in on the amplicon in the **Reference Coverage Chart** (see callout 3 in Figure 9).

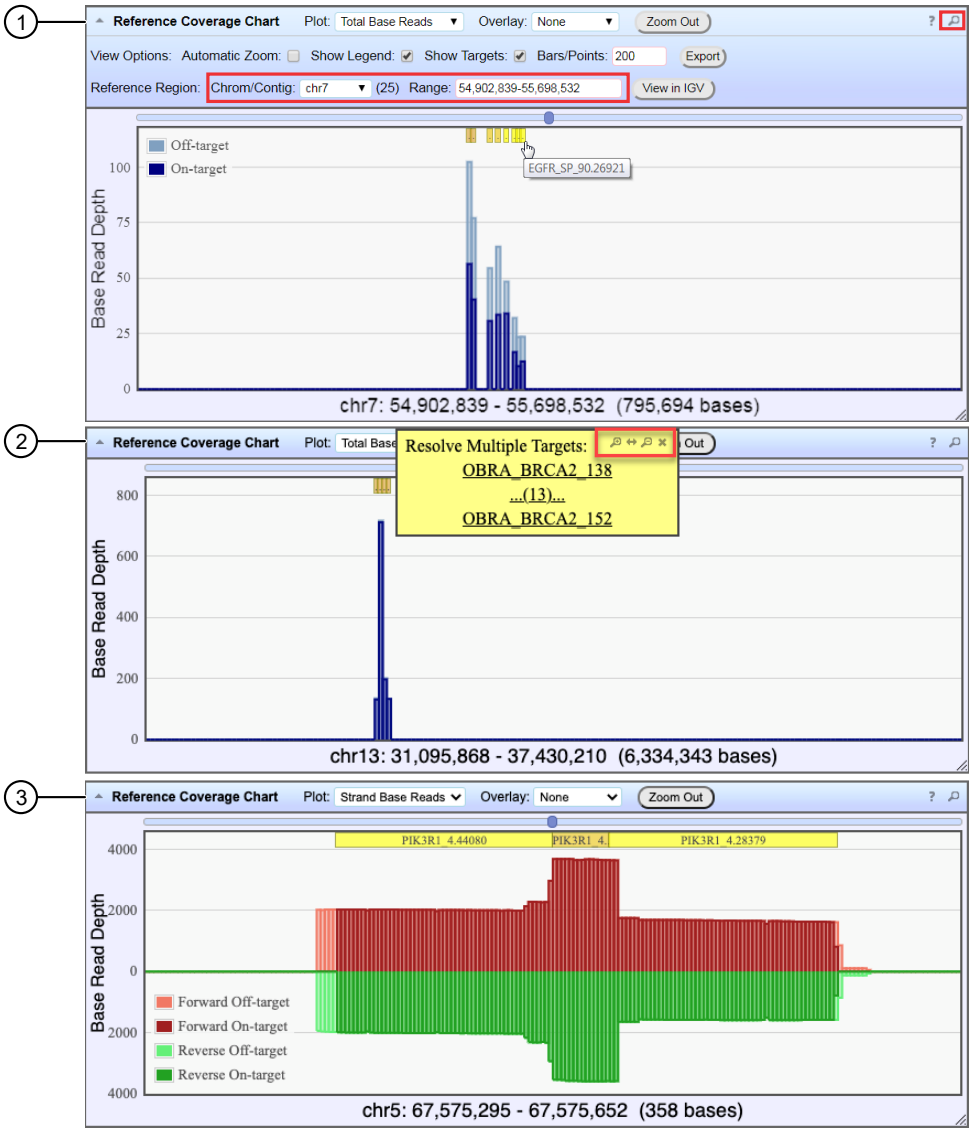


Figure 9 Representative Reference Coverage Chart

The Reference Coverage Chart is an overlay of where target regions are defined and overlap on the reference. The X-axis represents the target region chromosomal position. The Y-axis represents the Base Read Depth.

(1) Example Total Base Reads plot with Viewing Options panel expanded. The plot is zoomed in on a specific chromosomal region. Click a yellow bar (top of the chart) to open the detail pane for a specific amplicon in the Amplicon Coverage Chart (see callout 2 in Figure 8).

(2) Example Total Base Reads plot where a chromosomal region has multiple targets. In the Resolve Multiple Targets pane, click to zoom in on the region, or click one of the links to open the detail pane for the specific amplicon in the Amplicon Coverage Chart.

(3) Example Strand Base Reads plot that is zoomed in on one specific amplicon. Click the yellow box to open the detail pane for the specific amplicon in the Amplicon Coverage Chart.

Output files generated by the coverageAnalysis plugin

You can download coverageAnalysis 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 **(Help)** next to the file to open a description of the file.

The following is an example of the content of a results file that is generated by the coverageAnalysis plugin.

The list of files depends on the application type selected.

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 contains a 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 the following 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`: same as `base_cum_cov` but represented as a percentage of the total base reads.

(continued)

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 is the sum of `fwd_reads` and `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.
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.

(continued)

File	Description
Aligned reads BAM file	Contains all aligned reads that are 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).

G Oncomine™ Variant Annotator plugin criteria

The information in this appendix summarizes the criteria that is used by the Oncomine™ Variant Annotator plugin to find and annotate variants. The information shown in the Annotation Criteria columns is provided in VCF files. The variants are annotated for each listed variant type only if all of the conditions in the corresponding Annotation Criteria column are satisfied.

Oncomine™ tumor specific assays with Ion Reporter™ Software 5.16

Variant type	Oncomine™ Gene class	Oncomine™ Variant class	Annotation criteria
Copy number amplification	Gain-of-Function	Amplification	• SVTYPE = "CNV" • FILTER = "GAIN" • Occurs in a designated copy-gain gene
Copy number deletion	Loss-of-Function	Deletion	• SVTYPE = "CNV" • FILTER = "LOSS" • Occurs in a designated copy-loss gene
Copy number exon deletion	Loss-of-Function	ExonDeletion	• SVTYPE = "CNV" • FILTER = "LOSS" • SUBTYPE = "BigDel"
Long deletion	Loss-of-Function	LongDel	• Positive mutation call • SVTYPE = "LongDel"
Gene fusion	Gain-of-Function	Fusion	• SVTYPE = "Fusion" • FILTER = "PASS" • Is a targeted fusion isoform
RNA exon variant	Gain-of-Function	RNAExonVariant	• SVTYPE = "RNAExonVariant" or "Fusion" • FILTER = "PASS" • Is a targeted RNA exon variant

(continued)

Variant type	Oncomine™ Gene class	Oncomine™ Variant class	Annotation criteria
Expression imbalance	Gain-of-Function	ExpressionImbalance	• SVTYPE = "RNAExonTiles" • FILTER = "PASS" • Record meets Targeted Isoforms Detected Requirement
Loss-of-function truncating de novo mutation	Loss-of-Function Unclassified	Truncating	• Positive mutation call • Functional impact is frameshift block substitution, frameshift deletion, frameshift insertion, or nonsense • Occurs in a loss-of-function or unclassified gene
Missense hotspot mutation	Gain-of-Function Loss-of-Function Unclassified	Hotspot	• Positive mutation call • Functional impact is missense • Transcript and codon position occur in predefined missense hotspot list
In-frame hotspot mutation	Gain-of-Function Loss-of-Function Unclassified	Hotspot	• Positive mutation call • Function, transcript, and coding syntax occur in predefined in-frame hotspot list
Splice site hotspot mutation	Gain-of-Function Loss-of-Function Unclassified	Hotspot	• Positive mutation call • Transcript, location, and exon occur in predefined splice site hotspot list
Intronic hotspot mutation	Gain-of-Function Loss-of-Function Unclassified	Hotspot	• Positive mutation call • Transcript, location, and coding syntax occur in predefined intronic hotspot list
Promoter hotspot mutation	Gain-of-Function Loss-of-Function Unclassified	Hotspot	• Positive mutation call • Transcript, location, and coding syntax occur in predefined promoter hotspot list
Synonymous hotspot mutation	Gain-of-Function Loss-of-Function Unclassified	Hotspot	• Positive mutation call • Functional impact is synonymous • Transcript and coding syntax occur in predefined synonymous hotspot list
Gain-of-function truncating hotspot mutation	Gain-of-Function	Hotspot	• Positive mutation call • Function, transcript, and coding syntax occur in predefined truncating hotspot list • Occurs in a gain-of-function gene

(continued)

Variant type	Oncomine™ Gene class	Oncomine™ Variant class	Annotation criteria
Loss-of-function truncating hotspot mutation	Loss-of-Function	Truncating	• Positive mutation call • Function, transcript, and coding syntax occur in predefined truncating hotspot list • Occurs in a loss-of-function gene
MNV hotspot mutation	Gain-of-Function Loss-of-Function Unclassified	Hotspot	• Positive mutation call • Transcript and coding syntax occur in predefined MNV hotspot list
EGFR exon 19 deletion	Gain-of-Function	EGFRExon19Deletion	• Positive mutation call • Functional impact is nonframeshift deletion, nonframeshift block substitution • Deletion impacts codons 744–761 of EGFR
EGFR exon 20 insertion	Gain-of-Function	EGFRExon20Insertion	• Positive mutation call • Functional impact is nonframeshift insertion, nonframeshift block substitution • Insertion impacts codons 762–775 of EGFR or variant is COSM26720
ERBB2 exon 20 insertion	Gain-of-Function	ERBB2Exon20Insertion	• Positive mutation call • Functional impact is nonframeshift insertion, nonframeshift block substitution • Insertion impacts codons 770–783 of ERBB2
MET exon 14 skipping	Gain-of-Function	METExon14Skipping	• Positive mutation call • Location is splice site in MET exon 14, is intronic >= 4bp deletion in 30 nucleotides preceding MET exon 14, or variant is in MET Exon 14 Skipping confirmed list

Oncomine™ tumor specific assays with Ion Reporter™ Software 5.14

Variant type	Oncomine™ Gene class	Oncomine™ Variant class	Annotation criteria
Copy number amplification	Gain-of-Function	Amplification	• SVTYPE = "CNV" • FILTER = "GAIN" • Occurs in a designated copy-gain gene

(continued)

Variant type	Oncomine™ Gene class	Oncomine™ Variant class	Annotation criteria
Copy number deletion	Loss-of-Function	Deletion	• SVTYPE = "CNV" • FILTER = "LOSS" • Occurs in a designated copy-loss gene
Copy number exon deletion	Loss-of-Function	ExonDeletion	• SVTYPE = "CNV" • FILTER = "LOSS" • SUBTYPE = "BigDel"
Long deletion	Loss-of-Function	LongDel	• Positive mutation call • SVTYPE = "LongDel"
Gene fusion	Gain-of-Function	Fusion	• SVTYPE = "Fusion" • FILTER = "PASS" • Is a targeted fusion isoform
RNA exon variant	Gain-of-Function	RNA Exon Variant	• SVTYPE = "RNAExonVariant" or "Fusion" • FILTER = "PASS" • Is a targeted RNA exon variant
Expression imbalance	Gain-of-Function	Expression Imbalance	• SVTYPE = "RNAExonTiles" • FILTER = "PASS" • Record meets Targeted Isoforms Detected Requirement
Loss-of-function truncating de novo mutation	Loss-of-Function / Unclassified	Truncating	• Positive mutation call • Functional impact is frameshift block substitution, frameshift deletion, frameshift insertion, nonsense • Occurs in a loss-of-function or unclassified gene
Missense hotspot mutation	Gain-of-Function / Loss-of-Function / Unclassified	Hotspot	• Positive mutation call • Functional impact is missense • Transcript and codon position occur in predefined missense hotspot list
In-frame hotspot mutation	Gain-of-Function / Loss-of-Function / Unclassified	Hotspot	• Positive mutation call • Function, transcript, and coding syntax occur in predefined in-frame hotspot list
Splice site hotspot mutation	Gain-of-Function / Loss-of-Function / Unclassified	Hotspot	• Positive mutation call • Transcript, location, and exon occur in predefined splice site hotspot list

(continued)

Variant type	Oncomine™ Gene class	Oncomine™ Variant class	Annotation criteria
Intronic hotspot mutation	Gain-of-Function / Loss-of-Function / Unclassified	Hotspot	• Positive mutation call • Transcript, location, and coding syntax occur in predefined intronic hotspot list
Promoter hotspot mutation	Gain-of-Function / Loss-of-Function / Unclassified	Hotspot	• Positive mutation call • Transcript, location, and coding syntax occur in predefined promoter hotspot list
Gain-of-function truncating hotspot mutation	Gain-of-Function	Hotspot	• Positive mutation call • Function, transcript, and coding syntax occur in predefined truncating hotspot list • Occurs in a gain-of-function gene
Loss-of-function truncating hotspot mutation	Loss-of-Function	Truncating	• Positive mutation call • Function, transcript, and coding syntax occur in predefined truncating hotspot list • Occurs in a loss-of-function gene
MNV hotspot mutation	Gain-of-Function / Loss-of-Function / Unclassified	Hotspot	• Positive mutation call • Transcript and coding syntax occur in MNV hotspot list
EGFR exon 19 deletion	Gain-of-Function	EGFR Exon19 Deletion	• Positive mutation call • Functional impact is nonframeshift deletion, nonframeshift block substitution • Deletion impacts codons 744-761 of EGFR
EGFR exon 20 insertion	Gain-of-Function	EGFR Exon20 Insertion	• Positive mutation call • Functional impact is nonframeshift insertion, nonframeshift block substitution • Insertion impacts codons 762-775 of EGFR or variant is COSM26720
ERBB2 exon 20 insertion	Gain-of-Function	ERBB2 Exon20 Insertion	• Positive mutation call • Functional impact is nonframeshift insertion, nonframeshift block substitution • Insertion impacts codons 770-783 of ERBB2

(continued)

Variant type	Oncomine™ Gene class	Oncomine™ Variant class	Annotation criteria
MET exon 14 skipping	Gain-of-Function	METExon14 Skipping	• Positive mutation call • Location is splice site in MET exon 14, is intronic ≥4bp deletion in 30 nucleotides preceding MET exon 14, or variant is in MET Exon 14 Skipping confirmed list

Oncomine™ tumor specific assays with Ion Reporter™ Software 5.12

Variant type	Oncomine™ Gene class	Oncomine™ Variant class	Annotation criteria
Copy number amplification	Gain-of-Function	Amplification	• SVTYPE = "CNV" • FILTER = "GAIN" • Occurs in a designated copy-gain gene
Copy number deletion	Loss-of-Function	Deletion	• SVTYPE = "CNV" • FILTER = "LOSS" • Occurs in a designated copy-loss gene
Loss-of-function deleterious de novo mutation	Loss-of-Function / Unclassified	Deleterious	• Positive mutation call • Functional impact is frameshift block substitution, frameshift deletion, frameshift insertion, nonsense • Occurs in a loss-of-function or unclassified gene
Missense hotspot mutation	Gain-of-Function / Loss-of-Function / Unclassified	Hotspot	• Positive mutation call • Functional impact is missense • Transcript and codon position occur in predefined missense hotspot list
In-frame hotspot mutation	Gain-of-Function / Loss-of-Function / Unclassified	Hotspot	• Positive mutation call • Function, transcript, and coding syntax occur in predefined in-frame hotspot list
Splice site hotspot mutation	Gain-of-Function / Loss-of-Function / Unclassified	Hotspot	• Positive mutation call • Transcript, location, and exon occur in predefined splice site hotspot list

(continued)

Variant type	Oncomine™ Gene class	Oncomine™ Variant class	Annotation criteria
Intronic hotspot mutation	Gain-of-Function / Loss-of-Function / Unclassified	Hotspot	• Positive mutation call • Transcript, location, and coding syntax occur in predefined intronic hotspot list
Promoter hotspot mutation	Gain-of-Function / Loss-of-Function / Unclassified	Hotspot	• Positive mutation call • Transcript, location, and coding syntax occur in predefined promoter hotspot list
Truncating hotspot mutation	Gain-of-Function / Loss-of-Function / Unclassified	Hotspot	• Positive mutation call • Function, transcript, and coding syntax occur in predefined truncating hotspot list
MNV hotspot mutation	Gain-of-Function / Loss-of-Function / Unclassified	Hotspot	• Positive mutation call • Transcript and coding syntax occur in MNV hotspot list

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 sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

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

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.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

Related documentation

Document	Description
Ion AmpliSeq™ Library Kit Plus User Guide (Pub. No. MAN0017003)	Comprehensive instruction for the preparation of Ion AmpliSeq™ libraries and provides detailed instruction and troubleshooting for use of the Ion Library Equalizer™ Kit.
Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide (Pub. No. MAN0013432)	Describes the automated preparation and templating of Oncomine™ tumor specific panel libraries using the Ion Chef™ System.
Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide (Pub. No. MAN0016854)	Describes the automated template preparation of Oncomine™ tumor specific panel libraries using the Ion Chef™ System for sequencing on Ion GeneStudio™ S5 Systems.
Ion Reporter™ Software 5.16 User Guide (Pub. No. MAN0019148)	Describes features of the Ion Reporter™ Software.
IonReporterUploader Command-Line Utility User Guide (Pub. No. MAN0017648)	Describes command-line instruction for uploading large files to Ion Reporter™ Software.
Ion Library TaqMan™ Quantitation Kit User Guide (Pub. No. MAN0015802)	Provides detailed instruction and troubleshooting for use of the Ion Library TaqMan™ Quantitation Kit
Qubit™ dsDNA HS Assay Kits User Guide (Pub. No. MAN0002326)	Describes use of theQubit™ dsDNA HS Assay Kit to quantify DNA samples.
Demonstrated Protocol: Sample Quantification for Ion AmpliSeq™ Library Preparation Using the TaqMan™ RNAse P Detection Reagents Kit (pub. No. MAN0007732)	Provides detailed instruction for sample quantification using the TaqMan™ RNase P Detection Reagents Kit.

Note: For additional documentation, see “Customer and technical support” on page 137.

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**.