ion torrent

Oncomine™ Comprehensive Assay Plus

USER GUIDE

for use with:
Oncomine™ Comprehensive Assay Plus, DNA
Oncomine™ Comprehensive Assay Plus, RNA

Catalog Numbers A48577, A48578, A49667, and A49671

Publication Number MAN0018490

Revision G.0

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

ThermoFisher SCIENTIFIC

Life Technologies Corporation | 7335 Executive Way | Frederick, Maryland 21704 USA
For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: MAN0018490 G.0 (English)

Revision	Date	Description
G.0	26 September 2023	Updated the Ion AmpliSeq™ Chef Supplies DL8 (Part No. A29027) to include the PCR Plate Frame.
F.0	16 August 2023	Updated Ion Reporter™ Software 5.20 analysis workflows: • Oncomine™ Comprehensive Plus - w3.1 - DNA and Fusions - Single Sample • Oncomine™ Comprehensive Plus - w3.1 - DNA - Single Sample • Oncomine™ Comprehensive Plus - w3.1 - Fusions - Single Sample • Oncomine™ Comprehensive Plus - w3.1 - Annotate Variants - Single Sample • Support added for Genomic Instability Metric (GIM). • Section added to provide details about fusion detection methods.
E.0	28 June 2022	Updated Ion Reporter™ Software 5.18.4 analysis workflows: • Oncomine™ Comprehensive Plus - w2.4 - DNA and Fusions - Single Sample • Oncomine™ Comprehensive Plus - w2.4 - DNA - Single Sample • Oncomine™ Comprehensive Plus - w2.4 - Fusions - Single Sample • Oncomine™ Comprehensive Plus - w2.4 - Annotate Variants - Single Sample
D.0	17 June 2021	Updates: • BRCA visualization • Mutational Signature prediction report • Updated MSI analysis results • Updated Ion Reporter™ Software 5.18 analysis workflows: – Oncomine™ Comprehensive Plus - w2.2 - DNA and Fusions - Single Sample – Oncomine™ Comprehensive Plus - w2.2 - DNA - Single Sample – Oncomine™ Comprehensive Plus - w2.2 - Fusions - Single Sample – Oncomine™ Comprehensive Plus - w2.2 - Annotate Variants - Single Sample

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

Contents

1 Product information

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

Product description

The Oncomine™ Comprehensive Assay Plus is a targeted, next-generation sequencing (NGS) assay that provides a comprehensive genomic profiling solution appropriate for formalin-fixed paraffin-embedded (FFPE) tissues from solid tumors. The assay detects multiple biomarkers being investigated by cancer researchers. It allows concurrent analysis of DNA and RNA to simultaneously detect multiple types of variants in a single workflow, including single nucleotide variants (SNVs), insertions and deletions (indels), copy number variants (CNVs), BRCA1 and BRCA2 large genomic rearrangements (LGRs), genomic instability metric (GIM), microsatellite instability (MSI), tumor mutational burden (TMB), loss-of-heterozygosity (LOH), and gene fusions featuring FusionSync.

Features of the Oncomine™ Comprehensive Assay Plus include:

- Enables analysis of various single-gene variants, such as SNVs, indels, fusions, splice variants, and CNV, including both copy number gains and losses across 500+ genes.
- Detects complex biomarkers associated with TMB and MSI.
- Supports Homologous Recombination Deficiency (HRD) research by detecting mutations in 46 HRR genes, including BRCA LGRs, and assessing genomic scarring with the genomic instability metric (GIM).
- Robust performance from as little as 10 ng per pool of DNA/RNA isolated from FFPE samples including fine needle biopsies.
- Content driven by the Oncomine™ Knowledgebase and experienced scientists helps assure coverage of key targets aligned to published evidence.
- Streamlined bioinformatics analysis in Ion Reporter™ Software (or Genexus™ Software) optimized specifically for this assay.

The Oncomine™ Comprehensive Assay Plus, DNA panel includes the Ion AmpliSeq™ Sample ID Panel primers to prevent research sample misidentification and provide gender determination.

This guide covers manual library preparation from DNA and RNA using the Oncomine™ Comprehensive Assay Plus, DNA and Oncomine™ Comprehensive Assay Plus, RNA panels, respectively. Each assay panel can be used with barcoded adapters, so that up to 4 paired DNA and RNA samples and DNA and

RNA no-template controls (NTCs) can be combined and loaded onto a single Ion 550™ Chip in a single workflow to minimize the per-sample sequencing cost.

This guide also covers automated library preparation on the Ion Chef™ System using the Oncomine™ Comprehensive Assay Plus – Automated Library Preparation kit (Cat. Nos. A49667 and A49671). The kit provides the Oncomine™ Comprehensive Assay Plus, DNA (2-pools) and Oncomine™ Comprehensive Assay Plus, RNA (2-pools) at 2X concentration premeasured in barcoded primer pool tubes ready to load into an Ion AmpliSeq™ Chef Reagents DL8 cartridge.

Contents and storage

Oncomine™ Comprehensive Assay Plus – Manual Library Preparation

Oncomine™ Comprehensive Assay Plus (Cat. No. A48577) is designed to prepare barcoded sample libraries from DNA and RNA. The kits consist of the Oncomine™ Comprehensive Assay Plus, DNA, Manual Library Preparation panel (2-pool) (Part No. A45615) and the Oncomine™ Comprehensive Assay Plus, RNA, Manual Library Preparation panel (2-pool) (Part No. A45616), with two Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990). Sufficient reagents are provided to prepare libraries from 24 samples.

Contents	Amount	Storage
Oncomine™ Comprehensive Assay Plus, DNA, Manual Library Preparation		
2X DNA OCA Plus, Pool 1 of 2 (blue cap)	3 × 40 µL	–30°C to –10°C
2X DNA OCA Plus, Pool 2 of 2 (blue cap)	3 × 40 µL	
RMC	24 µL	
Oncomine™ Comprehensive Assay Plus, RNA, Manual Library Preparation		
5X RNA OCA Plus, Pool 1 of 2 (red cap)	3 × 16 µL	–30°C to –10°C
5X RNA OCA Plus, Pool 2 of 2 (red cap)	3 × 16 µL	
Ion AmpliSeq™ Library Kit Plus		
5X Ion AmpliSeq™ HiFi Mix (red cap)	2 x 120 µL	–30°C to –10°C
FuPa Reagent (brown cap)	2 x 48 µL	
Switch Solution (yellow cap)	96 µL	
DNA Ligase (blue cap)	2 x 48 µL	
25X Library Amp Primers (pink cap)	2 x 48 µL	
1X Library Amp Mix (black cap)	2 x 1.2 mL	
Low TE	2 x 6 mL	15°C to 30°C[1]

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

Oncomine™ Comprehensive Assay Plus, RNA – Manual Library Preparation

Oncomine™ Comprehensive Assay Plus, RNA – Manual Library Preparation (Cat. No. A48578) is designed to prepare barcoded sample libraries from RNA. The kits consist of the Oncomine™ Comprehensive Assay Plus, RNA – Manual Library Preparation panel (2-pool) (Part No. A45616), with one Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990). Sufficient reagents are provided to prepare libraries from 24 samples.

Contents	Amount	Storage
Oncomine™ Comprehensive Assay Plus, RNA – Manual Library Preparation		
5X RNA OCA Plus, Pool 1 of 2 (red cap)	3 × 16 µL	–30°C to –10°C
5X RNA OCA Plus, Pool 2 of 2 (red cap)	3 × 16 µL	
Ion AmpliSeq™ Library Kit Plus		
5X Ion AmpliSeq™ HiFi Mix (red cap)	120 µL	–30°C to –10°C
FuPa Reagent (brown cap)	48 µL	
Switch Solution (yellow cap)	96 µL	
DNA Ligase (blue cap)	48 µL	
25X Library Amp Primers (pink cap)	48 µL	
1X Library Amp Mix (black cap)	1.2 mL	
Low TE	6 mL	15°C to 30°C[1]

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

Oncomine™ Comprehensive Assay Plus – Automated Library Preparation

The Oncomine™ Comprehensive Assay Plus – Automated Library Preparation (Cat. No. A49667) provides the Oncomine™ Comprehensive Assay Plus, DNA, Chef-Ready panel (2-pool) (Part No. A45617) and Oncomine™ Comprehensive Assay Plus, RNA, Chef-Ready panel (2-pool) (Part No. A45618) at 2X concentration pre-measured in barcoded primer pool tubes ready to load into an Ion AmpliSeq™ Chef Reagents DL8 cartridge. In addition, the kit provides all the reagents and supplies in an Ion AmpliSeq™ Kit for Chef DL8 (Cat. No. A29024) sufficient for preparing 32 samples.

Note: For detailed information on preparing Oncomine™ Comprehensive Assay Plus libraries on the Ion Chef™ System, see the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432).

Component	Amount	Storage
Oncomine™ Comprehensive Assay Plus, DNA, Chef Ready		
2X DNA OCA Plus (pool 1 of 2)	4 × 150 µL	–30°C to –10°C
2X DNA OCA Plus (pool 2 of 2)	4 × 150 µL	
RMC	48 µL	
Oncomine™ Comprehensive Assay Plus, RNA, Chef Ready		
2X RNA OCA Plus (pool 1 of 2)	4 × 150 µL	–30°C to –10°C
2X RNA OCA Plus (pool 2 of 2)	4 × 150 µL	
Ion AmpliSeq™ Kit for Chef DL8		
Ion AmpliSeq™ Kit for Chef DL8 (Part No. A29025)	2 × 4 cartridges	–30°C to –10°C
Ion AmpliSeq™ Chef Solutions DL8 (Part No. A29026)	2 × 4 cartridges	2°C to 8°C[1]
Ion AmpliSeq™ Chef Supplies DL8 (per insert) (Part No. A29027) • Ion AmpliSeq™ Tip Cartridge L8 • PCR Plate Frame • PCR Frame Seal • Enrichment Cartridge	2 boxes with 4 inserts	15°C to 30°C
IonCode™ 0101–0132 in 96 Well PCR Plates (dried) (Part No. A29028) 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)	2 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.

Oncomine™ Comprehensive Assay Plus, RNA – Automated Library Preparation

The Oncomine™ Comprehensive Assay Plus, RNA – Automated Library Preparation (Cat. No. A49671) provides the Oncomine™ Comprehensive Assay Plus, RNA, Chef-Ready panel (2-pool) (Part No. A45618) at 2X concentration pre-measured in barcoded primer pool tubes ready to load into an Ion AmpliSeq™ Chef Reagents DL8 cartridge. In addition, the kit provides all the reagents and supplies in an Ion AmpliSeq™ Kit for Chef DL8 (Cat. No. A29024) sufficient for preparing 32 samples.

Note: For detailed information on preparing Oncomine™ Comprehensive Assay Plus libraries on the Ion Chef™ System, see the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432).

Component	Amount	Storage
Oncomine™ Comprehensive Assay Plus, RNA, Chef-Ready		
2X RNA OCA Plus (pool 1 of 2)	4 × 150 µL	–30°C to –10°C
2X RNA OCA Plus (pool 2 of 2)	4 × 150 µL	
Ion AmpliSeq™ Kit for Chef DL8		
Ion AmpliSeq™ Kit for Chef DL8 (Part No. A29025)	2 × 4 cartridges	–30°C to –10°C
Ion AmpliSeq™ Chef Solutions DL8 (Part No. A29026)	2 × 4 cartridges	2°C to 8°C[1]
Ion AmpliSeq™ Chef Supplies DL8 (per insert) (Part No. A29027) • Ion AmpliSeq™ Tip Cartridge L8 • PCR Plate Frame • PCR Frame Seal • Enrichment Cartridge	2 boxes with 4 inserts	15°C to 30°C
IonCode™ 0101–0132 in 96 Well PCR Plates (dried) (Part No. A29028) 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)	2 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.

Workflow overview—Manual library preparation

From manual library preparation to sequencing results report

Create a custom Planned Run in Torrent Suite™ Software (page 23)

Using a preinstalled Oncomine™ Comprehensive Assay Plus Planned Run template, set up a customized Planned Run template for reuse when the same conditions are used for multiple runs.

Create an assay-specific Planned Run in Torrent Suite™ Software (page 23)

Use a customized Planned Run template to create a Planned Run.

Manually prepare libraries (page 38)

Amplify the targets, partially digest the amplicons, amplify the libraries with barcoded primers, purify, then quantify the libraries using qPCR.

Combine libraries (page 53)

To sequence multiple barcoded libraries on a single chip, combine libraries before template preparation.

Template preparation and sequencing (page 56)

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

Analyze data (page 61)

Review results and visualize variants using Torrent Suite™ Software and Ion Reporter™ Software.

Workflow overview—Automated library preparation

From automated library preparation to sequencing results report

Create a custom Planned Run in Torrent Suite™ Software (page 23)

Using a preinstalled Oncomine™ Comprehensive Assay Plus Planned Run template, set up a customized Planned Run template for reuse when the same conditions are used for multiple runs.

Create an assay-specific Planned Run in Torrent Suite™ Software (page 25)

Use a customized Planned Run template to create a Planned Run.

Prepare libraries using the Ion Chef™ System (page 29)

- For RNA libraries, reverse transcribe the RNA, then load the Ion Chef™ Instrument.
- for DNA libraries, remove deaminated bases from the DNA, then load the Ion Chef™ Instrument.

Combine libraries (page 53)

To sequence multiple barcoded libraries on a single chip, combine libraries before template preparation.

Template preparation and sequencing (page 56)

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

Analyze data (page 61)

Review results and visualize variants using Torrent Suite™ Software and Ion Reporter™ Software.

2 Reagents, supplies, and required materials

This chapter lists the reagents, supplies, and materials needed to operate the and Ion GeneStudio™ S5 Series Sequencer, and provides consumables ordering and storage information. Reagents and supplies can be ordered as kits and starter packs, but most consumables can also be ordered individually as your needs require.

Note: Consumables that have catalog numbers are orderable. Components that have part numbers cannot be ordered individually.

Required materials not supplied

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
One of the following: IonCode™ Barcode Adapters 1-96 Kit Ion Xpress™ Barcode Adapters Kit	 A29747 4474517[1]
Ion Library TaqMan™ Quantitation Kit and real-time PCR system, see “Recommended materials” on page 15.	4468802 (A26121)
Agencourt™ AMPure™ XP Kit	NC9959336, NC9933872 **fisherscientific.com**
(RNA only) Ion Torrent™ NGS Reverse Transcription Kit	A45003
(DNA only) Uracil-DNA Glycosylase, heat-labile	78310100UN
One of the following thermal cyclers, or equivalent: • ProFlex™ 96-well PCR System • Veriti™ 96-Well Thermal Cycler • 2720 Thermal Cycler[2] • GeneAmp™ PCR System 9700 96-Well[2] or GeneAmp™ PCR System 9700 Dual 96-Well[2]	Various

(continued)

Item	Source
MicroAmp™ Optical 96-Well Reaction Plate or MicroAmp™ Optical 96-Well Reaction Plate with Barcode	N8010560 or 4306737
MicroAmp™ Fast Optical 96-Well Reaction Plate	4346907
MicroAmp™ Clear Adhesive Film	4306311
MicroAmp™ Optical Film Compression Pad	4312639
DynaMag™–96 Side Magnet or other plate magnet	12331D
Eppendorf™ DNA LoBind™ Microcentrifuge Tubes, 1.5 mL	13-698-791 **fisherscientific.com**
Nuclease-free Water	AM9932
Ethanol, Absolute, Molecular Biology Grade	BP2818500 **fisherscientific.com**
Pipettors, 2–200 µL, and low-retention filtered pipette tips	MLS

[1] Various kits are available. For more information, see **thermofisher.com**.
[2] Supported but no longer available for purchase.

Recommended materials

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
Recommended 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
96-well plate centrifuge	**MLS**
Qubit™ 4 Fluorometer[2]	Q33238

(continued)

Item	Source
Recommended for nucleic acid isolation	
MagMAX™ FFPE DNA/RNA Ultra Kit	A31881
Recommended for nucleic acid quantification	
Qubit™ dsDNA HS Assay Kit (DNA)	Q32851, Q32854,
Qubit™ RNA HS Assay Kit (RNA)	Q32852, Q32855
TaqMan™ RNase P Detection Reagents Kit	4316831

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

Recommended controls	Vendor	Part number
AcroMetrix™ Oncology Hotspot Control	Thermo Fisher Scientific	969056
Seraseq™ Tri-Level Tumor Mutation DNA Mix v2 HC	Seracare	0710-0097
Seraseq™ gDNA MSI-High Mix	Seracare	0710-1670
ATCC cell lines with CNV	**www.atcc.org**	ATCC® CRL-2327 ATCC® CRL-2336 ATCC® CRL-5868D
Seraseq™ gDNA TMB Mix Score 7	Seracare	0710-1326
Seraseq™ gDNA TMB Mix Score 9	Seracare	0710-1325
Seraseq™ gDNA TMB Mix Score 20	Seracare	0710-1324
Seraseq™ gDNA TMB Mix Score 26[1]	Seracare	0710-1323
Seraseq™ Fusion RNA Mix v4 18 RNA fusions: RET, ROS1, EGFRvIII, EGFR, ALK, NTRK3, FGFR3, NTRK1, METex14, PPARG1, BRAF, ERG	Seracare	0710-0497
Seraseq™ FFPE NTRK Fusion RNA Reference Material	Seracare	0710-1031
Horizon™ ALK RET ROS RNA fusion RNA fusions: EML4-ALK, CCDC6-RET, and SLC34A2-ROS1	Horizon	HD784
CancerSeq™ Plus Paraffin Tissue Curl (5 curls) Copy number variation (CNV): CCNE1, EGFR, ERBB2, GNAS, KRAS, RB1 (-)	BioChain	T2235152-SC Lot No. B906046

[1] The TMB Score is generated using whole-exome sequencing and does not reflect targeted NGS panel-based TMB Scores. For more information, see the data sheet provided by the manufacturer.

3 Before you begin

Procedural guidelines

- Minimize freeze-thaw cycles of Oncomine™ Comprehensive Assay Plus panels and the RMC by aliquoting into low bind tubes as needed for your experiments. Panels can be stored at 4°C for 1 year. Store RMC at –30°C to –10°C.
- 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.
- 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.

Before each use of the kit

- Thaw components that contain enzymes—such as 5X Ion AmpliSeq™ HiFi Mix, FuPa Reagent, DNA Ligase, and 1X Library Amp Mix —on ice, and keep on ice during procedure. All other components, including primer pools, can be thawed at room temperature. Gently vortex and centrifuge before use.
- If there is visible precipitate in the Switch Solution after thawing, vortex or pipet up and down at room temperature to resuspend.
- Bring the Agencourt™ AMPure™ XP Reagent to room temperature.

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

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 if available, 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 of DNA if available, for manual library preparation and automated library preparation. Increasing the amount of DNA results in higher-quality libraries, especially when DNA quality or quantity is unknown.

4 Create a Planned Run

IMPORTANT! This kit is compatible with Torrent Suite™ Software 5.18 or later and Ion Reporter™ Software 5.20 or later. Before proceeding, we recommend that you update to the latest available versions of Torrent Suite™ Software, Ion Reporter™ Software, and Ion Chef™ System software. Contact your service representative for help with upgrading the software.

Note: The IonReporterUploader 5.20 plugin must be installed in Torrent Suite™ Software. If your Ion Reporter™ Software account is not configured, configure it through the software settings (⚙▾) see "Configure the IonReporterUploader plugin in Torrent Suite™ Software" on page 105).

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 S5™ XL Sequencer, Ion GeneStudio™ S5 Plus Sequencer, or Ion GeneStudio™ S5 Prime 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 system help for Torrent Suite™ Software, which is available by clicking **Help** in the software.

In Torrent Suite™ Software 5.18 or later, use the **Oncomine™ Comprehensive Plus – DNA and Fusions** template as the primary Planned Run template for the Oncomine™ Comprehensive Assay Plus.

Application category in Torrent Suite™ Software	Template name	Description
Oncology – Solid Tumor	Oncomine™ Comprehensive Plus – DNA and Fusions	DNA and RNA Planned Run template
	Oncomine™ Comprehensive Plus – DNA	DNA-only Planned Run template
	Oncomine™ Comprehensive Plus – Fusions[1]	RNA-only Planned Run template

[1] Available in Torrent Suite™ Software 5.16 or later.

Update Oncomine™ Comprehensive Assay Plus templates in Torrent Suite™ Software

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™ Comprehensive Assay Plus, then in that row click **Update**.

The software update begins automatically and when finished shows a status of **Complete** in the **Update** column.

Create a custom Planned Run template

IMPORTANT! Before creating a Planned Run, you might need to enable the Oncomine™ Comprehensive Plus templates and upload the **Reference Library** and the **Target Regions** and **Hotspots** BED files on the Ion Torrent™ Server. For more information, see Appendix B, "Supplemental information". Contact your local service representative to obtain the most current files.

We recommend setting up a customized Planned Run template for reuse when the same conditions will be used for multiple runs. For more information on how to create a new Planned Run, see the Torrent Suite™ Software help system.

Note: Before a Planned Run is created, Copy Number Variation (CNV), microsatelite instability (MSI), and tumor mutational burden (TMB) functionalities might require that the Ion Reporter™ Software analysis workflow that includes user-defined optimizations be copied and edited. For more information, see "CNV baseline creation" on page 115, "MSI parameters" on page 90), and "Set TMB Classification parameters" on page 105, or refer to the Ion Reporter™ Software system help.

1. Sign in to the Torrent Suite™ Software.
2. In the **Plan** tab, in the **Templates** screen, click **Oncology – Solid Tumor** in the left navigation menu.
3. In the list of templates, find **Oncomine™ Comprehensive Plus – DNA and Fusions**, then click ⚙ **(Actions) ▸ 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[2]	Select **OCAPlus.20221214.designed.bed.**

[1] Fusions Reference Library and Fusions Target Regions are not necessary for analysis in Torrent Suite™ Software.
[2] Check with your service representative for updates to ensure that the most current files are used.

5. Click the **Ion Reporter** step, then select your Ion Reporter™ Software account (version 5.20 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 105). For instructions on how to install the IonReporterUploader plugin, see the Torrent Suite™ Software help system.

6. In the **Existing Workflow** dropdown list, select the appropriate Ion Reporter™ Software analysis workflow (page 58) for your Planned Run (for example, **Oncomine™ Comprehensive Plus - w3.1 - DNA and Fusions - Single Sample**), then click **Next**.

7. In the **Research Application** step, verify that the appropriate **Research Application** and **Target Technique** are pre-selected, then click **Next**.

8. In the **Kits** step, verify that **Ion Chef** is selected for the **Template Kit**, then complete the following fields 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	Ion 550™ Kit – Chef	
Sequencing Kit	Ion S5™ Sequencing Kit	
Chip Type	Ion 550™ Chip	
Barcode Set	IonXpress or IonCode	IonCode
Flows	500	

9. Select or edit the optional information fields as appropriate for your run, then click **Next**.

10. In the **Plugins** step, ensure that the **sampleID** and **coverageAnalysis** plugins are selected.

11. 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**.

12. In the **Projects** step, select the project appropriate to your run, then click **Next**.

13. 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 **Oncology – Solid Tumor** application.

Create a Planned Run for manual library preparation

1. Sign in to the Torrent Suite™ Software.
2. In the **Plan** tab, in the **Templates** screen, click **Oncology – Solid Tumor** in the left navigation menu.
3. In the list, click on your customized Planned Run template name, or click ⚙ **(Actions)** ▸ **Plan Run**. The **Create Plan** workflow opens to the **Plan** step.
4. Enter a name for the plan in the **Run Plan Name** field.
5. Make or confirm the following selections:

Field	Action
Analysis Parameters	Ensure that **Default (Recommended)** is selected.
Use same reference & BED files for all barcodes	Ensure that this checkbox is selected.
Same sample for DNA and Fusions?	Ensure that this checkbox is selected.
Number of barcodes	Enter the number of barcodes, then click ✔ button to the right of this field. The standard number of barcodes for an Oncomine™ Comprehensive Assay Plus DNA and Fusions run is 8.
Sample Tube Label	Enter or scan the barcode of the Ion Chef™ Library Sample Tube to be used in the run.
Chip Barcode	No entry required.
Oncology	Ensure that this is selected.
Pre-implantation Genetic Screening	Ensure that this is deselected.

6. Enter the sample and barcode information:

Field[1]	Action
Barcode	Use the default barcode selections, or select the barcode for each sample from the dropdown list.
Sample Name	Each sample must have a unique name. Type in the field to overwrite the default name. We recommend that you use unique names even between runs.
Control Type (expanded)	Select **No Template Control** from the dropdown list to designate a sample as a no template control.
Sample ID	*(Optional)* Click in the field, then enter a sample ID.
Sample Description	*(Optional)* Click in the field, then enter a sample description.

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Field[1]	Action
Annotations (expanded)	Click to reveal **Cancer Type** and **Cellularity %**.
Cancer Type	Select from the dropdown list. Click to copy the entry to all the rows.
Cellularity %	The percentage of tumor cells in the sample. This is a whole number between 1 and 100. Each DNA sample ***must*** have a cellularity percentage. This field is optional for RNA samples. Click to copy the same entry to all rows. Method of calculation: Auto-calculated tumor cellularity (default) or Manually input Tumor Cellularity.[2] **Note:** • If the auto-calculated or manual tumor cellularity is less than 40%, 40% is used for the gene CNV adjustment. • The calculated/user input cellularity are displayed, even if CNV calls were adjusted to 40% cellularity. • When auto-calculated Tumor Cellularity Percentage fails, the manual Tumor Cellularity Percentage is used. If the manual Tumor Cellularity Percentage is not available, 100% Tumor Cellularity Percentage is used for gene CNV. [3]
Ion Reporter Workflow	Ensure the correct workflow is selected. **Note:** If the workflows are not displayed in the dropdown list, select the **Show All Workflows** checkbox to view all workflows on the selected Ion Reporter™ Server.
Relation	Ensure the correct value is auto-populated. Select from the dropdown list to change.
Gender	Select from the dropdown list. Click to copy the entry to all the rows. **Note:** This is a required field to perform CNV analysis. By default Ion Reporter™ Software treats samples as female. Gender needs to be specified in the run plan for male samples. Otherwise, CN losses falsely are called for genes in X-chromosome.
IR Set ID[4]	The **IR Set ID** links individual samples for analysis. Ensure the correct value is autopopulated. Select from the dropdown list to change. **IMPORTANT!** Ensure that a unique value is entered for each sample.

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

[2] If tumor cellularity percentage is determined to be not reliable by auto-calculation, then "Manual" is displayed.

[3] Manual Tumor Cellularity Percentage may not be available if the sample is not uploaded from Torrent Suite™ Software.

[4] 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).

7. 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.

Create a Planned Run for automated library preparation using sample sets

Automated Oncomine™ Comprehensive Assay Plus library preparation on the Ion Chef™ System requires use of sample sets for creating your planned run. For more information on creating a sample set, see “Create a sample set to prepare 2 pools of 4 libraries each” on page 29.

If combining more than one sample set, each sample set must correspond to Oncomine™ Comprehensive Assay Plus automated library preparations and use the same barcode kit to be included in a single Planned Run.

Note: Ensure the Planned Run Template to be used has the connection to Ion Reporter™ Software account configured and the desired analysis workflow selected.

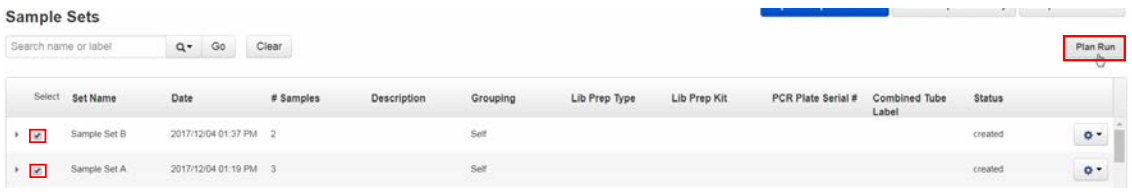
---

1. In the **Plan** tab, in the **Samples** screen, find the Sample Sets that you want to add to the Planned Run.

2. Select one or more Sample Set (for example a DNA Sample Set and an RNA Sample Set) to add to the Planned Run.

   - To plan a run using one Sample Set, click ⚙ **(Actions)** ▸ **Plan Run** in the row of the Sample Set.

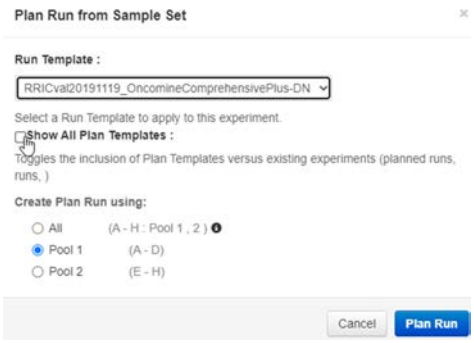
   - To plan a run using multiple Sample Sets, select the checkboxes next to the Sample Sets you want to add to the Planned Run, then click **Plan Run**.



IMPORTANT! Ensure that all Sample Sets used in the Planned Run use the same barcode kit. To verify the barcode kit used, expand the Sample Set entry to view its details.

The **Select a Run Template to apply to this experiment** dialog box lists Planned Run templates that support your Sample Set.

3. Select a Run Template to use for the experiment. Select the samples to Create Plan Run using: All (A–H: Pool 1, 2), Pool 1 (A–D), or Pool 2 (E–H) then click **Plan Run**.



Note: If you do not see the template that you are looking for, select **Show All Templates**, then look again for the template.

The Create Plan workflow bar opens to the **Barcoding** step with the Sample Sets that you selected:

4. In the **Barcoding** step in the workflow bar, make or confirm the following selections.

Field	Action
Analysis Parameters	Ensure that **Default (Recommended)** is selected.
Use same reference & BED files for all barcodes	Ensure that this checkbox is selected.
Same sample for DNA and Fusions?	Ensure that this checkbox is selected.
Number of barcodes	The standard number of barcodes for an Oncomine™ Comprehensive Assay Plus DNA and Fusions run is 8 without no-template controls (NTC) and 7 with NTCs. Enter the number of barcodes, then click ✔ button to the right of this field.
Sample Tube Label	Enter or scan the barcode of each Ion Chef™ Library Sample Tube that will be used in the run.
Chip Barcode	No entry required.
Oncology	Ensure that this is selected.
Pre-implantation Genetic Screening	Ensure that this is deselected.

Note: System-installed run templates do not have Ion Reporter™ Software accounts configured and do not display the sample set information.

5. (*If needed*) Click the **Ion Reporter** step in the workflow bar.

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 105). For instructions on how to install the IonReporterUploader plugin, see the Torrent Suite™ Software help system.

a. Select your Ion Reporter™ Software account (version 5.20 or later).

b. In the **Existing Workflow** dropdown list, select the appropriate Ion Reporter™ analysis workflow, then click **Next**.

c. In the **Ion Reporter Upload Options**, select **Automatically upoad to an Ion Reporter after run completion**.

d. Click the **Barcoding** step in the workflow bar, alternatively click **Next** 4 times to advance to the **Barcoding** step.

Note: Sample information should now be automatically populated in the **Samples Table**.

6. In the **Barcoding** step, make sure your sample set information has been correctly imported. Edit entries in each field as needed in the **Samples Table**, then click **Next**.

Field[1]	Description
Barcode	Use the imported barcode selections, or select the barcode for each sample from the dropdown list.
Sample Name (required)	Each sample must have a unique name. Type in the field to overwrite the default name. We recommend that you use unique names even between runs.
Control Type (expanded)	Click on the **Control Type** column header to expand the **Control Type** column. Select **No Template Control** from the dropdown list to designate a sample as a no template control.
Sample ID	*(Optional)* Click in the field, then enter a sample ID.
Sample Description	*(Optional)* Click in the field, then enter a sample description.
DNA/Fusions	For DNA and Fusions application, select **DNA** or **Fusions** from the dropdown menu for each samples.
Annotations (expanded)	Click to reveal **Cancer Type** and **Cellularity %**.
Cancer Type	Select from the dropdown list. Click to copy the entry to all the rows.
Cellularity %	Each DNA sample ***must*** have a cellularity percentage. This field is optional for RNA samples. Click to copy the same entry to all rows.
Ion Reporter Workflow	Ensure the correct workflow is selected. **Note:** If the workflows are not displayed in the dropdown list, select the **Show All Workflows** checkbox to view all workflows on the selected Ion Reporter™ Server.
Relation	Select sample relationship group.

(continued)

Field[1]	Description
Gender	Select "Male", "Female", or "Unknown" from the dropdow list. Click to copy the entry to all the rows. **Note:** Gender must be defined to perform CNV analysis. By default "Unknown" is treated as female.
IR Set ID[2]	The **IR Set ID** links individual samples for analysis. Ensure the correct value is autopopulated. Set the IR Set ID to the same value for related samples. **IMPORTANT!** Ensure that a unique value is entered for each sample. Do not give unrelated samples the same IR Set ID value even if the value is zero or blank.

[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).

Note: You can save the samples table to a CSV file as a template for future use. Click **Save Samples Table** above the upper right corner of the **Samples Table** to save the CSV file to your computer.

7. Review the **Plugins** and **Projects** tabs, make selections appropriate to your run, then click **Next**.
8. In the **Save & Finish** step in the workflow bar, enter a name for the Planned Run in the provided field.
9. Click **Save & Finish**.

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.

5 Automated library preparation on the Ion Chef™ System

This chapter describes library preparation using the following components:

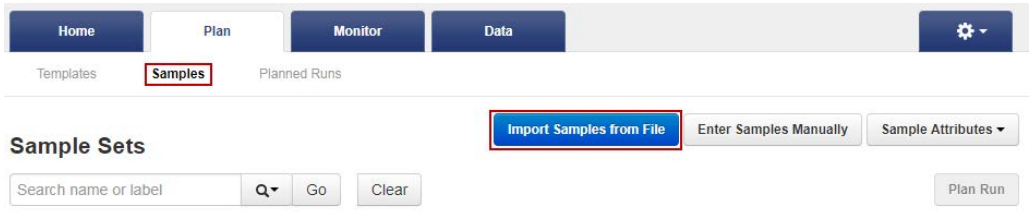
- Oncomine™ Comprehensive Assay Plus, DNA, Chef-Ready Library Preparation (Part No. A45617)
- Oncomine™ Comprehensive Assay Plus, RNA, Chef-Ready Library Preparation (Part No. A45618)
- Ion Torrent™ NGS Reverse Transcription Kit (Cat No. A45003)
- Uracil-DNA Glycosylase, heat-labile (Cat No. 78310100UN)
- Ion AmpliSeq™ Kit for Chef DL8 (Cat No. A29024)

Create a sample set to prepare 2 pools of 4 libraries each

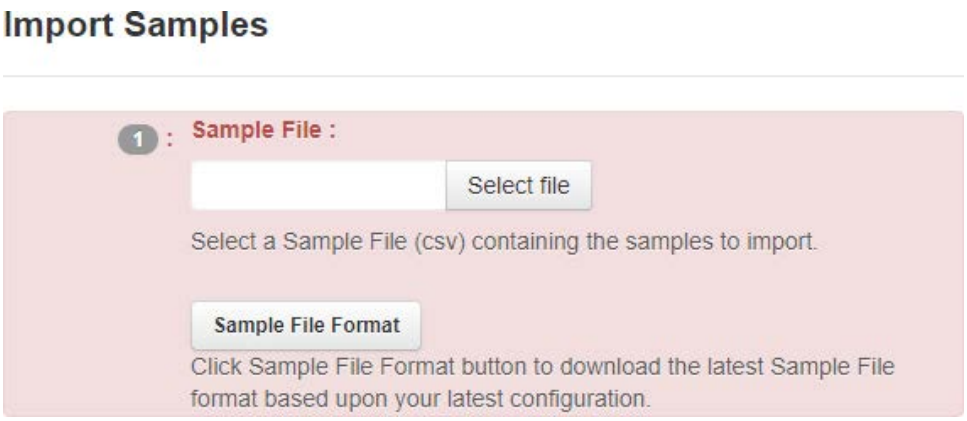
IMPORTANT! The Oncomine™ Comprehensive Assay Plus requires selection of the **2 Library Pools - OCA Plus** Library prep Protocol that limits the number of libraries to 4 per pool in order to generate sufficient read depth when sequencing.

Note: In Torrent Suite™ Software 5.16 or later Sample Sets are required for automated library preparation. You can import new samples into Torrent Suite™ Software with the **Import Samples from File** feature, using a CSV template file that is available in Torrent Suite™ Software to simplify the process. During this process, you can also create a new Sample Set for the new samples.

1. In the **Plan** tab, click **Samples**, then click **Import Samples from File**. For more information, see the Torrent Suite™ Software help system.



2. In section 1 of the **Import Samples** page, click **Sample File Format** to download a sample CSV template.



The sample file format CSV contains the version of the CSV file in the top row, and sample attributes in separate columns.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W
1	CSV Version (required)	2																					
2	Sample Name (required)	Sample ID	Control Type	PCR Plate Position	Barcodekit	Barcode	Gender	Type	Group	Description	Sample Collection Date	Sample Receipt Date	Nucleotide Type	Sample Source	Panel Pool Type	Cancer Type	Cellularity %	Biopsy Days	Cell Number	Couple ID	Embryo ID	Population	Mouse Strains
3	Sample-1			A	IonCode	IonCode_0101	Female	Tumor					DNA	FFPE	Dual Pool		50						
4	Sample-2			B	IonCode	IonCode_0102	Male	Tumor					RNA	FFPE	Dual Pool		50						
5	NTC-1		No Template Control	C	IonCode	IonCode_0103																	

3. Fill out the template CSV file as completely as possible, then save it to the location of your choice.

Note:

- Required columns include: Sample name, PCR Plate Position (A–H), Barcode Kit (IonCode Barcodes 1–32), and Barcode.
- Recommended columns include: Sample ID, Gender, Type (sample type, such as self), Group (number that indicates the sample is a single sample, pair or trio), DNA/RNA, Cancer Type, and Cellularity %.

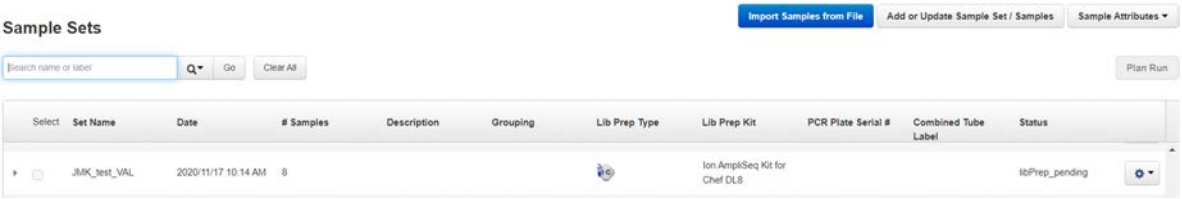
4. When the CSV file is filled out and saved, click **Select File**, navigate to the completed CSV file, then click **Open**.

5. In section 2 of the **Import Samples** page, click **Add Sample Set**, then enter or select the required information in each field.
   a. Enter a Sample Set Name.
   b. Select the Group Type.
   c. Select the Library Prep Type—**AmpliSeq on Chef**.
   d. Select the Library Prep Kit—**Ion AmpliSeq Kit for Chef DL8**.

e. Select the Library Prep Protocol—**2 Library Pools - OCA Plus**.

Note: Completed libraries will be delivered to uncapped library Recovery Tubes in Position C (samples A–D) and Position D (samples E–H) in the Ion AmpliSeq™ Chef Reagents DL8 cartridge. Save the caps. To run 8 RNA samples simultaneously on the same chip do not select a Library Prep Protocol. All 8 sample libraries are combined in a single Recovery Tube v2 in Position D of the Ion AmpliSeq™ Chef Reagents DL8 cartridge.

6. Click **Save & Finish**.
   The software automatically imports the samples into the **Sample Sets** table.



Saved sample sets that enable 2 library pools for OCA Plus can then be selected on the Ion Chef™ Instrument user interface when setting up Ion AmpliSeq™ Kit for Chef DL8 library preparation runs.

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.

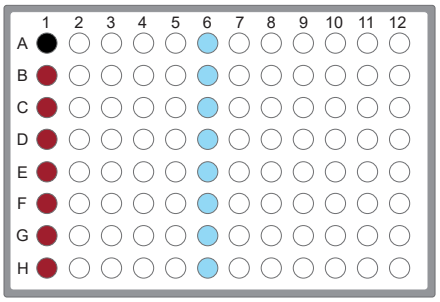
1. If the RNA was prepared from FFPE tissue and not previously heat-treated, heat at 80°C for 10 minutes, then cool to room temperature.

2. Remove and discard the plate seal from an IonCode™ 96-well PCR Plate.

3. 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 and balance the number of positive samples between rows A–D and E–H.
- 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.
- If processing RNA samples that are to be combined with a paired DNA library ensure the samples are processed in the correct rows A–D or E–H.

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

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.

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

Temperature	Time
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.

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

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

8. 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 20 times before sealing the plate.

   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.

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™ Comprehensive Assay Plus 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 33.

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 Oncomine™ Comprehensive Assay Plus libraries on the Ion Chef™ System.

IMPORTANT! When starting the library preparation run on the Ion Chef™ Instrument ensure that the correct Kit Type and Sample set are selected, and that **2 Library Pools - OCA Plus** is displayed in order to properly prepare Oncomine™ Comprehensive Assay Plus–automated libraries. If 2 Library Pools - OCA Plus is not displayed the default library preparation script is run which results in all 8 libraries combined into a single pool. The 2 Library Pools - OCA Plus library preparation option is only available in Planned Runs created with Torrent Suite™ Software 5.16 or later.

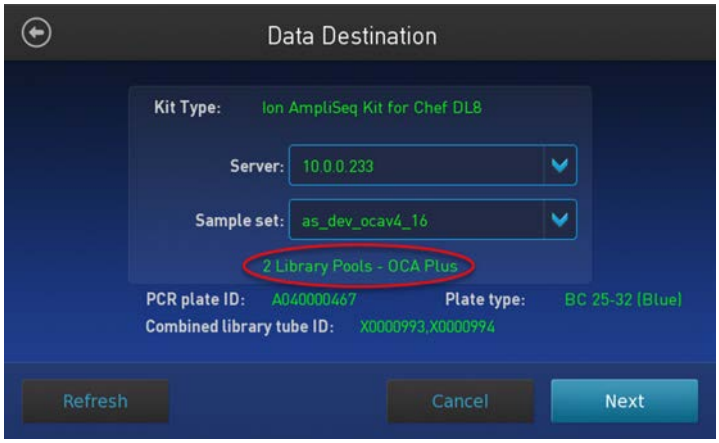


Figure 1 Example of a correct Oncomine™ Comprehensive Assay Plus setup

Ensure that *2 Library Pools - OCA Plus* appears below the Sample set dropdown list.

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 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.

Automated DNA library preparation

RMC in DNA target amplification reactions

Oncomine™ Comprehensive Assay Plus has been developed to support a wide range of biomarkers, including assessment of microsatellite instability (MSI). MSI arises from defects in the mismatch repair (MMR) system and is associated with hypermutability of short DNA sequence repeats (microsatellite locations) throughout the genome.

RMC is composed of in-sample standards that function as internal references in the analysis pipeline to ensure the robustness of MSI assessment in case of variations in sample preparation or run conditions. RMC is added to the DNA target amplification reaction.

Remove deaminated bases from FFPE DNA

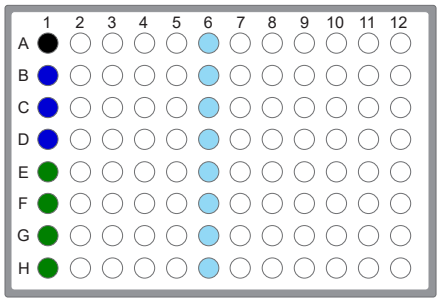
Sample age, storage conditions, and FFPE preservation methods can lead to significant cytosine deamination of the isolated DNA. This deamination can result in an artificially high deamination score when determining the tumor mutational burden result. We have demonstrated that deaminated cytosine (uracil) bases can be enzymatically removed by treatment with Uracil DNA Glycosylase (UDG).

Note: We recommend treating all samples including FFPE and high quality (for example, commercial controls or DNA isiolated from cell lines) DNA with UDG to remove deaminated bases before target amplification.

1. Remove and discard the plate seal from an IonCode™ Barcode Adapters 96-well PCR plate.
2. For each FFPE DNA sample, add the following components to a single well in column 1 of the IonCode™ Barcode Adapters 96-well PCR plate.

Component	Volume
20 ng FFPE DNA[1]	≤9 µL
Uracil-DNA Glycosylase, heat-labile	1 µL
Low TE	to 10 µL

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



- Column 1 wells contains 20 ng of FFPE DNA sample in 10 µL, or 10 µL Nuclease-free Water as non-template control. Samples A–D delivered to uncapped library Recovery Tube in Position C in the Ion AmpliSeq™ Chef Reagents DL8 cartridge.
- Column 1 wells contains 20 ng of FFPE DNA sample in 10 µL, or 10 µL Nuclease-free Water as non-template control. Samples E–H delivered to uncapped library Recovery Tube in Position D in the Ion AmpliSeq™ Chef Reagents DL8 cartridge.
- *(Optional)* 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 10 µL of Nuclease-free Water as non-template control into column 1 wells that do not contain a DNA sample and balance the number of positive samples between rows A–D and E–H.
- We recommend processing at least 6 samples per run. We do not recommend processing 1, 2 or 5 samples per run. If you do process 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.
- If processing only 3 or 4 samples, group them together either in rows A–D or E–H.

3. Mix the reaction by pipetting at least half the total volume up and down at least 5 times, then seal the plate with MicroAmp™ Clear Adhesive Film. Alternatively, seal the plate, vortex for 5 seconds to mix the reactions, then centrifuge briefly to collect the contents.

IMPORTANT! To prevent evaporation during UDG treatment, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate. Offset the film to the left so that the adhesive does not cover the barcode label.

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

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

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

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

6. Carefully remove the plate seal, then add the following components to each well.

Note: If processing multiple samples, prepare a reaction master mix (+ 5–10% overage), then add 5 µL to each well.

Component	Volume
RMC	1.5 µL
Nuclease-free water	3.5 µL
Total volume per well (including sample)	**15 µL**

7. Mix the reaction by pipetting at least half the total volume up and down at least 5 times, then carefully inspect each well for air bubbles. Remove any air bubbles by gentle pipetting. Alternatively, seal the plate with MicroAmp™ Clear Adhesive Film, vortex for 5 seconds to mix the reactions, then centrifuge briefly to collect the contents.

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™ Comprehensive Assay Plus 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 DNA library preparation” on page 37.

Ion Chef™ Instrument setup information for automated DNA library preparation

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

IMPORTANT! When starting the library preparation run on the Ion Chef™ Instrument ensure that the correct Kit Type and Sample set are selected, and that **2 Library Pools - OCA Plus** is displayed in order to properly prepare Oncomine™ Comprehensive Assay Plus–Chef Ready libraries. If 2 Library Pools - OCA Plus is not displayed the default library preparation script is run which results in all 8 libraries combined into a single pool. This exceeds the capacity of the Ion 550™ Chip. The 2 Library Pools - OCA Plus library preparation option is only available in Planned Runs created with Torrent Suite™ Software 5.16 or later.

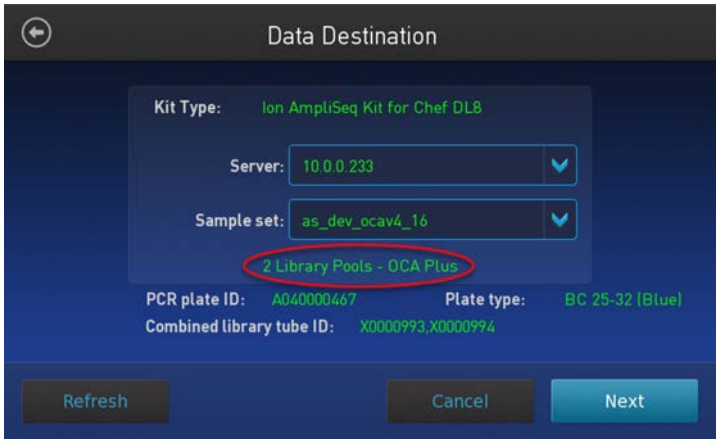


Figure 2 Example of a correct Oncomine™ Comprehensive Assay Plus setup

Ensure that *2 Library Pools - OCA Plus* appears below the Sample set dropdown list.

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

Stating material	# of primer pools	Target amplification cycles	Anneal & extension time
High quality DNA[1]	2	13	16 minutes
FFPE DNA[1]	2	16	16 minutes

[1] Due to the disparity in the required number of target amplification cycles for high quality and FFPE DNA we do NOT recommend running both high quality and FFPE samples on the same plate using the same input amount.

6 Manual library preparation

This chapter describes library preparation using the following kits:

- Oncomine™ Comprehensive Assay Plus, DNA, Manual library preparation (Part No. A45615)
- Oncomine™ Comprehensive Assay Plus, RNA, Manual library preparation (Part No. A45616)
- Ion AmpliSeq™ Library Kit Plus (Cat No. 4488990)
- Ion Torrent™ NGS Reverse Transcription Kit (Cat No. A45003)
- Uracil-DNA Glycosylase, heat-labile (Cat No. 78310100UN)

RNA preparation and cDNA amplification

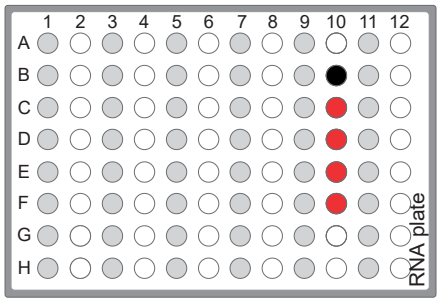
Use the components of the Oncomine™ Comprehensive Assay Plus, RNA (Cat. No. A45616) for the follow procedures.

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.

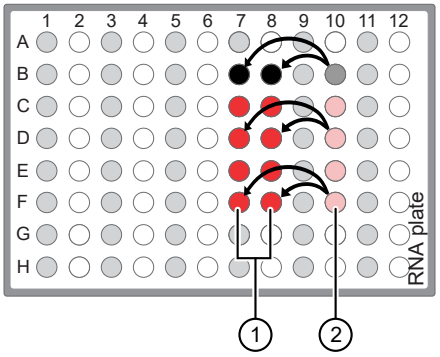
1. Place the 96-well plate in a pre-chilled cold block or on ice.

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™ Comprehensive Assay Plus, RNA 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 Table 1	Denature	98°C	15 sec
	Anneal and extend	60°C	4 min (RNA Panel)
Hold	—	10°C	Hold

Table 1 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.

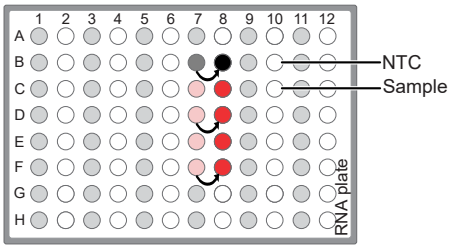
[3] The recommended number of cycles is based on 10 ng RNA input per primer pool. Adjust the cycle number for lower or higher RNA input.

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 preparing both RNA and DNA sample libraries 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.



DNA preparation and amplification

Use the components of the Oncomine™ Comprehensive Assay Plus, DNA, Manual Library Preparation (Cat. No. A47620) for the following procedures.

RMC in DNA target amplification reactions

Oncomine™ Comprehensive Assay Plus has been developed to support a wide range of biomarkers, including assessment of microsatellite instability (MSI). MSI arises from defects in the mismatch repair (MMR) system and is associated with hypermutability of short DNA sequence repeats (microsatellite locations) throughout the genome.

RMC is composed of in-sample standards that function as internal references in the analysis pipeline to ensure the robustness of MSI assessment in case of variations in sample preparation or run conditions. RMC is added to the DNA target amplification reaction.

Remove deaminated bases from FFPE DNA

Sample age, storage conditions, and FFPE preservation methods can lead to significant cytosine deamination of the isolated DNA. This deamination can result in an artificially high deamination score when determining the tumor mutational burden result. We have demonstrated that deaminated cytosine (uracil) bases can be enzymatically removed by treatment with Uracil DNA Glycosylase (UDG). For this reason, we recommend treating DNA isolated from FFPE samples with UDG to remove deaminated bases before target amplification.

Note: We recommend treating all samples including FFPE and high quality (for example, commercial controls or DNA isiolated from cell lines) DNA with UDG to remove deaminated bases before target amplification.

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

Component	Volume
20 ng FFPE DNA[1]	≤5.5 µL
Uracil-DNA Glycosylase, heat-labile	1 µL
Low TE	to 6.5 µL

[1] Do not exceed 40 ng maximum FFPE DNA sample as input. Substitute an equal volume of nuclease-free water or low TE to prepare a no-template control (NTC).

2. Mix the reaction by pipetting at least half the total volume up and down at least 5 times, then seal the plate with MicroAmp™ Clear Adhesive Film. Alternatively, seal the plate, vortex for 5 seconds to mix the reactions, then centrifuge briefly to collect the contents.

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

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

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

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

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

5. Carefully remove the plate seal, then proceed immediately to “Prepare DNA target amplification reactions” on page 44, adding the target amplification reaction components to the well containing 6.5 µL of UDG treated FFPE DNA.

Prepare DNA target amplification reactions

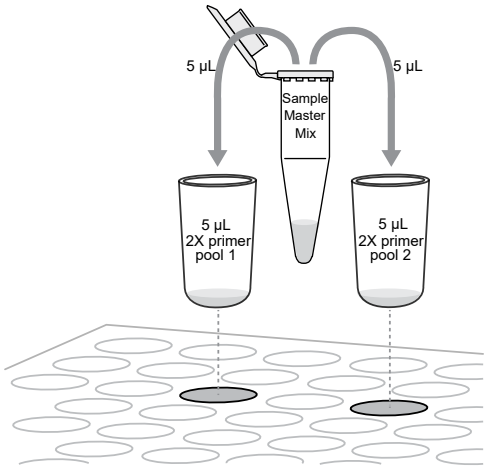
IMPORTANT! Primer pools and 5X Ion AmpliSeq™ HiFi Mix are viscous. Pipet slowly and mix thoroughly.

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) (treated with UDG)	6.5 µL
RMC	1 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™ Comprehensive Assay Plus, DNA 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 DNA targets” .

Amplify the DNA targets

IMPORTANT! When amplifying multiple samples in a single PCR plate, make sure 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 Table 2	Denature	99°C	15 sec
	Anneal and extend	60°C	16 min (DNA Panel)
Hold	—	10°C	Hold

Table 2 Recommended cycle number

Input nucleic acid[1]	Recommended number of cycles[2]	Cycle number adjustment[3]	
	10 ng DNA input	1 ng DNA input	20 ng DNA input
High quality DNA	12	+3	–1
FFPE DNA	15	+3	–1

[1] If both high quality and FFPE nucleic acids are being used in the same reaction, use the FFPE 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 DNA input per primer pool. Adjust the cycle number for lower or higher DNA input.

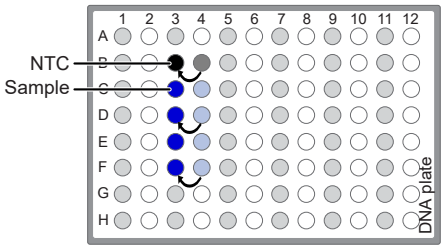
STOPPING POINT Target amplification reactions may be stored at 10°C overnight on the thermal cycler. For longer periods, store at –20°C.

Combine the DNA target amplification reactions

Note: Perform the following steps on ice or in a pre-chilled 4°C cold block.

1. Remove the DNA plate from the thermal cycler, then centrifuge briefly to collect the contents.
2. Carefully remove the plate seal.
3. For each sample, combine both 10-µL DNA target amplification reactions into a single well.

IMPORTANT! Accurate volume transfer in this step is critical. We recommend using a single-channel pipettor. If you are using a multi-channel pipettor, visually check pipette tips to ensure that volumes are equivalent.



Note: In multi-zone thermal cyclers ensure samples are in the same zone for amplification.

The total volume for each sample should be ~20 µL.

Library preparation

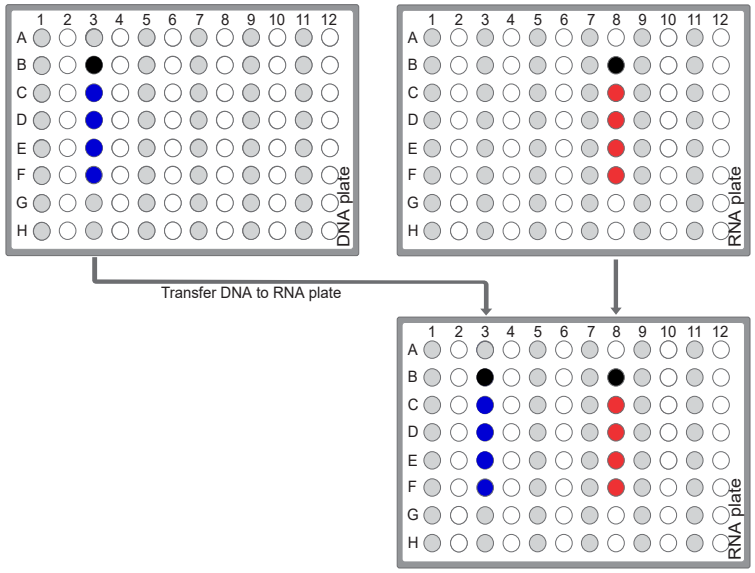
Transfer the DNA amplicons

If preparing both DNA and cDNA libraries with the same FuPa digestion conditions, library preparations can be transferred to a single plate for simultaneous processing. For more information, see "Partially digest the DNA and cDNA amplicons" on page 47.

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

Partially digest the DNA and cDNA amplicons

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

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
50°C	20 min (cDNA/DNA)
55°C	20 min (cDNA/DNA)
60°C	20 min (cDNA/DNA)
10°C	Hold (for up to 1 hour)

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.

We recommend IonCode™ Barcode Adapters.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 (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.
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.

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.

   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 a 1.5-mL Eppendorf LoBind™ tube for long-term storage.

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 51.

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 these steps for quantifying your Oncomine™ Comprehensive Assay Plus libraries.

1. Prepare three 10-fold serial dilutions of the *E. coli* DH10B Ion Control Library (~68 pM, from the 2) 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[1,2]	9 µL	4.5 µL

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

[2] High quality samples may require further dilution (5–10 fold) to give results within the range of the prepared standards. We recommend starting with 1000X dilution for RNA library quantification.

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 100.

8. Proceed to “Combine 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 Combine libraries, Template, and Sequence

Combine libraries

When comparing genomic DNA and RNA libraries that are prepared from the same sample, unequal volumes of libraries can be combined to produce different read depths for the paired DNA and RNA libraries.

- Combine DNA and RNA library pools from the same samples prepared on an Ion Chef™ Instrument.

Note: Upon completion of the Ion Chef™ Instrument run libraries have already been adjusted to a final concentration of ~100 pM each and pooled on the Ion Chef™ Instrument. Libraries from samples A–D are in the Recovery Tube in Position C and samples E–H in Position D of the Ion AmpliSeq™ Chef Reagents DL8 cartridge.

a. Combine the DNA library pool with the RNA library pool for a given set of 4 samples at a 90:10 ratio (DNA:RNA—18 µL of DNA library pool + 2 µL of RNA library pool).

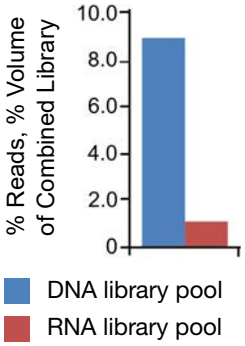
Note: This ratio differs from previous Oncomine™ Comprehensive Assay protocols.

IMPORTANT!

- The combined DNA and RNA library pools must be from matched Recovery Tubes. For example, combine Position C libraries, do NOT combine sample DNA libraries from Position C (samples A–D) with sample RNA libraries from Position D (samples E–F) or vice versa.
- Each combined library must have a unique barcode.

b. Dilute the combined (90:10 DNA:RNA) pooled libraries 2-fold (~50 pM final concentration).

Note: We recommend sequencing up to 4 research samples (one library pool, either samples A–D or E–H) on a single Ion 550™ Chip.



Library pool	Sample	Barcode	Fractional volume (90:10 DNA:RNA)	
DNA–C	DNA-1	BC_0101	0.225	0.9
	DNA-2	BC_0102	0.225	
	DNA-3	BC_0103	0.225	
	DNA-4	BC_0104	0.225	
RNA–C	RNA-1	BC_0109	0.025	0.1
	RNA-2	BC_0110	0.025	
	RNA-3	BC_0111	0.025	
	RNA-4	BC_0112	0.025	
Sum	—	—	**1.0**	**1.0**

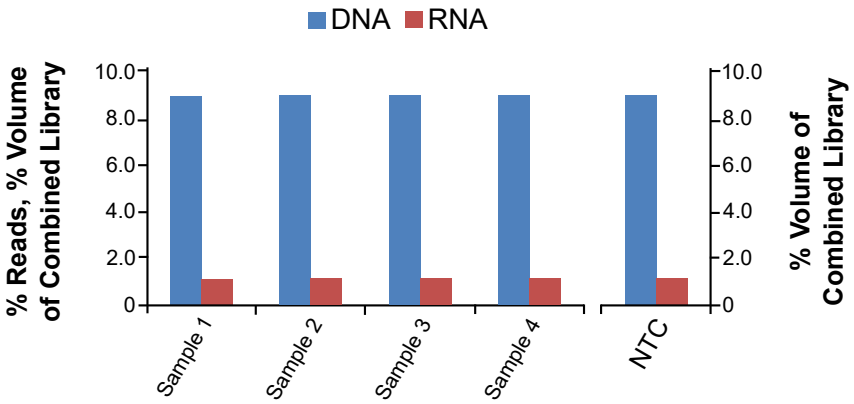
- Combine manually prepared DNA and RNA libraries.
  a. Combine each uniquely barcoded DNA and RNA library (~50 pM each) from the same sample at an 90:10 ratio (DNA:RNA—18 µL of DNA library + 2 µL of RNA library).

Note: This ratio differs from previous Oncomine™ Comprehensive Assay protocols.

b. Combine equal volumes of the paired libraries (90:10 DNA:RNA) to be sequenced on the same chip.

Note:

- We recommend sequencing up to 5 samples (4 research samples plus 1 no-template control) on a single Ion 550™ Chip.
- For runs that include a no-template control (NTC), add in the same fractional volumes of undiluted DNA and RNA NTC libraries as is added for equivalent sample libraries.



Sample	Barcode	Fractional volume (90:10 DNA:RNA)
DNA-1	BC_0101	0.18
RNA-1	BC_0102	0.02
DNA-2	BC_0103	0.18
RNA-2	BC_0104	0.02
DNA-3	BC_0105	0.18
RNA-3	BC_0106	0.02
DNA-4	BC_0107	0.18
RNA-4	BC_0108	0.02
DNA-5 (NTC)	BC_0109	0.18
RNA-5 (NTC)	BC_0110	0.02
Sum	—	**1.0**

- Combine only manually prepared RNA libraries to be sequenced on the same chip.

Note: We recommend sequencing up to 32 sample RNA libraries on a single Ion 550™ Chip.

a. Combine 2 µL of each uniquely barcoded RNA library (~50 pM each), then mix thoroughly.

- Combine only RNA library pools prepared on an Ion Chef™ Instrument to be sequenced on the same chip.

  Note:
  - When combining only RNA libraries, library pools in the Recovery Tube in Position C (samples A–D) can be combined with library pools in the Recovery Tube in Position D (samples E–F).
  - We recommend sequencing up to 32 sample RNA libraries on a single Ion 550™ Chip or Ion 540™ Chip.
  - We recommend sequencing up to 8 sample RNA libraries on a single Ion 530™ Chip.

  a. Combine 5 µL from each of 4 pooled RNA libraries (~100 pM each).

  b. Mix thoroughly, then dilute the combined library pools 2-fold (~50 pM final concentration).

- Combine only manually prepared DNA libraries to be sequenced on the same chip.

  Note: We recommend sequencing up to 4 sample DNA libraries on a single Ion 550™ Chip.

  a. Combine 10 µL of each uniquely barcoded DNA library (~50 pM each).

- Combine only DNA library pools prepared on an Ion Chef™ Instrument to be sequenced on the same chip.

  Note: We recommend sequencing up to 4 sample DNA libraries on a single Ion 550™ Chip. Upon completion of the Ion Chef™ Instrument run each Recovery Tube contains 4 pooled libraries adjusted to a final concentration of ~100 pM.

  a. Dilute 15 µL of the pooled DNA libraries 2-fold (~50 pM final concentration), then mix thoroughly.

Guidelines for templating and sequencing

Proceed to template preparation and sequencing using the following kits.

Chip	Template System	Sequencer	Kit	User Guide
Ion 550™ Chip	Ion Chef™ System	Ion S5™ XL Sequencer, Ion GeneStudio™ S5 Plus Sequencer, or Ion GeneStudio™ S5 Prime Sequencer	Ion 550™ Kit – Chef (Cat. No. A34541)	*Ion 550™ Kit – Chef User Guide* (Pub. No. MAN0017275)
			Ion 550™ Single Chip Supplemental Kit (Cat. No. A36953)	

To create a specific Run Plan for use in templating and sequencing, see Chapter 4, “Create a Planned Run”. Refer to the appropriate user guide listed in the table for more information.

8 Variant analysis

Analysis workflows in Ion Reporter™ Software

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 to manually launch an analysis, see "Manually launch a DNA analysis" on page 60 and "Manually launch a Fusions analysis" on page 60.

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

Ion Reporter™ Software 5.20 or later includes the following analysis workflows:

Workflow name[1]	Description
Oncomine™ Comprehensive Plus - w3.1 - DNA and Fusions - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted Ion AmpliSeq™ DNA manual or Ion Chef™ automated libraries, computes automatic tumor cellularity, Loss-of-Heterozygosity, TMB and microsatellite instability (MSI), as well as gene fusions from targeted Ion AmpliSeq™ RNA manual or Ion Chef™ automated libraries, from Oncomine™ Comprehensive Assay Plus run on the Ion 550™ Chip. TMB uses the TMB (Non-Germline Mutations) filter chain with TMB algorithm v4.0. MSI status is computed using a baseline with MSI algorithm v4.0.3. Released with: Ion Reporter™ Software 5.20. Workflow Version: 3.0. Supports Homologous Recombination Deficiency (HRD) research by detecting mutations in 46 HRR genes, including BRCA LGRs, and assessing genomic scarring with the genomic instability metric (GIM).
Oncomine™ Comprehensive Plus - w3.1 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs), computes automatic tumor cellularity, Loss-of-Heterozygosity, TMB and microsatellite instability (MSI) from targeted Ion AmpliSeq™ DNA manual or Ion Chef™ automated libraries from Oncomine™ Comprehensive Assay Plus run on the Ion 550™ Chip. TMB uses the TMB (Non-Germline Mutations) filter chain with TMB algorithm v4.0. MSI status is computed using a baseline with MSI algorithm v4.0.3. Released with: Ion Reporter™ Software 5.20. Workflow Version: 3.0. Supports Homologous Recombination Deficiency (HRD) research by detecting mutations in 46 HRR genes, including BRCA LGRs, and assessing genomic scarring with the genomic instability metric (GIM).
Oncomine™ Comprehensive Plus - w3.1 - Fusions - Single Sample	Detects and annotates gene fusions from targeted Ion AmpliSeq™ RNA manual or Ion Chef™ automated libraries from Oncomine™ Comprehensive Assay Plus run on the Ion 550™ Chip. Released with: Ion Reporter™ Software 5.20. Workflow Version: 3.0. Supports Homologous Recombination Deficiency (HRD) research by detecting mutations in 46 HRR genes, including BRCA LGRs, and assessing genomic scarring with the genomic instability metric (GIM).
Oncomine™ Comprehensive Plus - w3.1 - Annotate Variants - Single Sample	Annotates VCFs from the Oncomine™ Comprehensive Assay Plus. Released with: Ion Reporter™ Software 5.20. Workflow Version: 3.0.

[1] Workflow names can vary depending on the version of Ion Reporter™ Software.

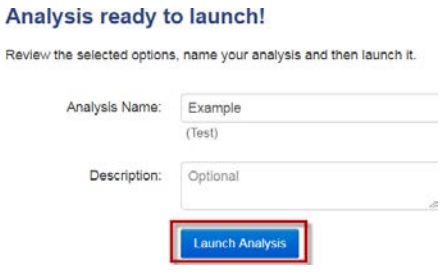
Manually launch a DNA and Fusions analysis

1. Sign in to 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 list of workflows, select the checkbox in the row that contains the appropriate workflow name (for example, Oncomine™ Comprehensive Plus - w3.1 - DNA and Fusions - Single Sample), then click **Actions** ▸ **Launch Analysis** in the **Details** pane.
5. Select a DNA sample.
   a. In the **Samples** step, search by any unique identifier you used to label your samples during setup, then ensure that the sample **Percentage Cellularity**, **Sample Type** and **Gender** are defined.

      Note:
      - We recommend that you define the sample **Gender** to perform accurate CNV analysis. By default the sample is treated as female.
      - By default the software uses the auto-calculated **Percentage Cellularity** value. In the rare case that the **Percentage Cellularity** can not be automatically calculated, the software will use the user-entered value.

   b. Click the checkbox to select a DNA sample, then click **Add samples**.
6. Select an RNA sample.
   a. In the **Samples** step, search by any unique identifier you used to label your samples during setup, then ensure the sample **Sample Type** is defined.
   b. Click the checkbox to select an RNA sample, then click **Add samples**.
7. Enter a **Group Name** for the sample group, then click **Add to Analysis**.
8. In the **Plugins** step, ensure that the **Oncomine™ Variant Annotator 3.3** plugin is selected, then click **Next**.
9. *(Optional)* Enter an **Analysis Name** and **Description**.
10. Click **Launch Analysis**.

    If you selected multiple samples, a separate analysis is launched for each sample for single-sample analysis workflows.

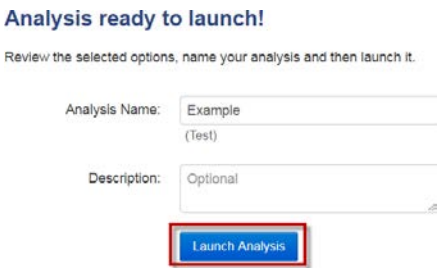


The analysis is added to the list in the **Analyses** tab.

Manually launch a DNA analysis

1. Sign in to Ion Reporter™ Software.
2. In the **Workflows** tab, in the **Overview** screen, select **DNA** 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, select the checkbox next to the appropriate workflow (for example, Oncomine™ Comprehensive Plus - w3.1 - 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 **Percentage Cellularity**, **Sample Type** and **Gender** are defined.

   Note: We recommend that you define the sample Gender to perform accurate CNV analysis. By default this is treated as female. In the rare instance that the **Percentage Cellularity** can not be auto-calculated the software will use the user entered value. By default the software uses the auto-calculated **Percentage Cellularity** value.
6. Click the checkbox to select a DNA sample, then click **Next**.
7. In the **Plugins** step, ensure that the **Oncomine™ Variant Annotator 3.3** plugin is selected, then click **Next**.
8. *(Optional)* Enter an **Analysis Name** and **Description**.
9. Click **Launch Analysis**.

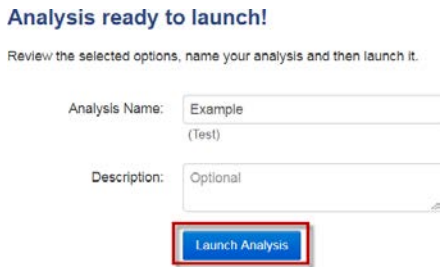


The analysis is added to the list in the **Analyses** tab.

Manually launch a Fusions analysis

1. Sign in to Ion Reporter™ Software.
2. In the **Workflows** tab, in the **Overview** screen, select **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, select the checkbox next to the appropriate workflow (for example, Oncomine™ Comprehensive Plus - w3.1 - Fusions - 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 that the **Sample Type** is defined.
6. Click the checkbox to select a fusions sample, then click **Next**.
7. In the **Plugins** step, ensure that the **Oncomine™ Variant Annotator 3.3** plugin is selected, then click **Next**.
8. *(Optional)* Enter an **Analysis Name** and **Description**.
9. Click **Launch Analysis**.



The analysis is added to the list in the **Analyses** tab.

View results

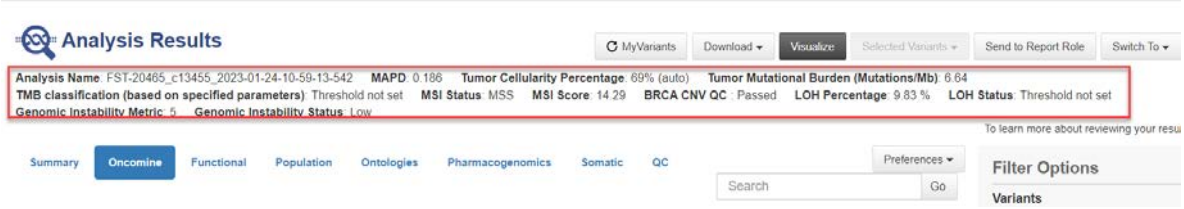
Ion Reporter™ Software analyses are performed automatically on uploading of the data files from the Torrent Suite™ Software.

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 more 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 to select a row (do not click the hyperlink in the Analysis or Sample columns) 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** screen.

The **Analysis Results** screen opens to the **Oncomine** tab, and shows only variants relevant to cancer. Metrics listed at the top of the screen are described in the following table.



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.
Sample ID	If the sample data are 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.
Tumor Cellularity Percentage	The percent of tumor cells in the sample. Method of calculation: Auto-calculated tumor cellularity (default) or Manually input Tumor Cellularity input per user.[1] **Note:** • If the auto-calculated or manual tumor cellularity is less than 40%, 40% is used for the gene CNV adjustment. • The calculated/user input cellularity are displayed, even if CNV calls were adjusted to 40% cellularity. • When auto-calculated Tumor Cellularity Percentage fails, the manual Tumor Cellularity Percentage is used. If the manual Tumor Cellularity Percentage is not available, 100% Tumor Cellularity Percentage is used for gene CNV. [2]
Tumor Mutational Burden (Mutations/Mb)	The value of the result for the tumor mutational burden calculation.

(continued)

Item	Description
TMB Classification (based on specified parameters)	• High—The Tumor Mutational Burden (Mutations/Mb) is greater than the value entered in the **TMB-High Threshold** parameter. • Low—The Tumor Mutational Burden (Mutations/Mb) is less than the value entered in the **TMB-Low Threshold** parameter. • Intermediate—The Tumor Mutational Burden (Mutations/Mb) is equal to or greater than the value entered in the **TMB-Low Threshold** parameter AND less than or equal to the value entered in the **TMB-High Threshold** parameter. • Threshold not set—TMB classification thresholds settings were not included in the analysis workflow or were set incorrectly.
MSI Status	• MSI-High—MSI score is greater than the threshold value set in the MSI-High Threshold parameter. • MSS—MSI score is less than the threshold value set in the MSS Threshold parameter. • No Call—MSI score is equal to or greater than the value entered in the **MSS Threshold** parameter AND less than or equal to the value entered in the **MSI- ThresholdHigh** parameter. • QCFail—Indicates determination of MSI status was not reliable due to the MSI baseline. For more information, see "MSI QC Failure" on page 102 or for help contact support (see "Customer and technical support" on page 132).
MSI Score	A sample-level MSI score that is calculated with individual MSI marker scores. The overall score is used to determine the MSI status of the sample.
BRCA CNV QC	A quality control metric that is based on the MAPD threshold. BRCA CNV QC is PASSED or FAILED. If failed, a reason is provided in a message.
LOH Percentage	Genomic loss-of-heterozygosity (sample level LOH percentage (%LOH)) is the percentage of genomic segments with (LOH) detected. Calculated as the sum of the sizes of the genomic segments with LOH detected / total size of genomic segments assessed for LOH.
LOH Status	Threshold values for LOH Status are not provided in the pre-defined analysis workflow. The analysis workflow always reports the LOH Status as **Threshold not set** unless a user sets the threshold to **High** or **Low** in the parameter settings.
Genomic Instability Metric	A proprietary measurement that quantifies genomic scarring associated with Homologous Recombination Deficiency (HRD). It summarizes unbalanced copy number changes in autosomes that are determined using genomic segmentation, and generates a value between 0–100. Higher GIM values correlate with the observation of more genomic instability in the sample.
Genomic Instability Status	A status for the Genomic Instability Metric with one of the following values. • **High**—The value for a GIM equal to the threshold or higher. • **Low**—The value for a GIM less than the threshold. • **Threshold not set**—The value when the threshold is not set. • **NOCALL**—The value shown when GIM cannot be calculated. **Note:** The default threshold of 16 is based on ovarian cancer.

[1] If tumor cellularity percentage is determined to be not reliable by auto-calculation, then "Manual" is displayed.

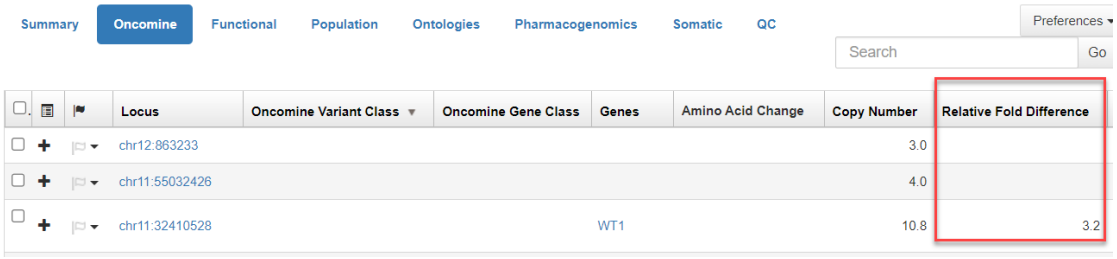
[2] Manual Tumor Cellularity Percentage may not be available if the sample is not uploaded from Torrent Suite™ Software.

6. In the **Analysis Results** table, sort or filter the data with filter chains, or with annotations for use withOncomine™ assays.
   a. In the **Filter Options** pane, select the desired **Filter Chain**.

   Note:
   - The default **Filter Chain** is **Oncomine™ Extended (5.20)**. This filter chain includes all Oncomine™-annotated variants and variants that may be relevant to cancer due to their inclusion in one or more of the following classes: CNV variants with FILTER value of GAIN or LOSS, likely somatic mutations based on dbSNP, 5000Exomes, ExAC, and UCSC Common SNPs annotation source databases, and variants with ClinVar annotations of pathogenic or likely pathogenic. Each variant that is called must meet all the conditions of the filter chain to be filtered-in. If another filter chain is applied and the analysis is saved, changes to the variant calls that are saved in the VCF file and can affect downstream workflows.
   - Select **No Filter** to view all the variant calls attempted by the variant caller.
   - For more information about filter chains, see the Ion Reporter™ Software help system.

   b. In the **Oncomine** tab, click the column headers to sort the list of variants by **Oncomine Variant Class** or **Oncomine Gene Class**. See the Ion Reporter™ Software help system for more options.

   Oncomine™ Comprehensive Assay Plus workflows use a new parameter, relative fold difference (RFD), to adjust copy-number gain calls relative to the arm-level changes. RFD enables distinguishing between gene CNV calls due to arm-level and focal copy number changes. For more information, see “Relative Fold Difference” on page 112.



   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 a final report” on page 93), or download the analysis files (see “Download Ion Reporter™ Software annotation files” on page 94). 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 detection methods

Note: For fusion variants detected by expression imbalance alone (RNAExonTiles), we recommend verifying the result using an orthogonal method.

The Oncomine™ Comprehensive Assay Plus uses the targeted panel design in combination with software algorithms to detect known and novel gene fusion isoforms. The assay employs the following methods to detect fusions.

Targeted method

In the targeted fusion detection method, panel primers are designed to target specific exon-exon junctions of fusions where the driver gene, the partner gene, and the breakpoint between the driver and the partner gene are known. The sequencing reads are mapped to a reference file that contains only the known gene fusions.

Non-targeted method

In the non-targeted fusion detection method, the panel primers are used to detect fusions between novel combinations of known driver and partner genes. The sequencing reads are mapped to a broader reference, such as the whole exome. Mapping the reads to a broader reference allows for the detection of multiple configurations of driver and partner genes as well as detection of novel breakpoints between the known partner and driver genes.

Low confidence non-targeted fusions are identified in VCF output files with key value pairs as follows.

Table 3 Key value pairs for non-targeted fusions

Type of non-targeted fusion identified	Definition	Key value pair
Out-of-frame sequence	The expected non-targeted sequence from the amplicon reference file is used to check whether the sequence is in frame. If the non-targeted sequence is out of frame (compared to the original partner or driver genes), the key-value pair `NT_FUSION_IN_FRAME=FALSE` is included in the VCF file.	`NT_FUSION_IN_FRAME` Values: • `FALSE` • `TRUE` f the key value pair is not included, then `NT_FUSION_IN_FRAME` is assumed to be `TRUE`, indicating that an out-of-frame sequence is not found.

Table 3 Key value pairs for non-targeted fusions *(continued)*

Type of non-targeted fusion identified	Definition	Key value pair
Partner-partner fusion	Non-targeted fusions are identified if one of the genes in an isoform is not a driver gene and another is (i.e., partner-driver fusion). The input reference BED file includes annotations for driver genes, for example, in the case of EML4-ALK.E6A19.COSF1296.2, the DRIVER_GENE=ALK. In cases where the `DRIVER_GENE` field is missing in the BED file, the software checks whether at least one of the two genes involved in the non-targeted fusion is a 3p gene.	`NT_FUSION_DRIVER_INVOLVED` Values: • `FALSE` • `TRUE` If the key value pair is not included, then `NT_FUSION_DRIVER_INVOLVED` is assumed to be `TRUE`, indicating that a partner-partner fusion is not found.
Secondary isoform fusion	In some samples, a non-targeted fusion isoform might be detected in a sample while additional targeted isoforms with the same gene pair are also detected (that is, isoforms that are defined in the panel). In this case, the non-targeted fusion isoform should be identified as `NT_FUSION_SECONDARY=TRUE`, if its read count coverage is below ***<5%*** of the reads of any of the other isoforms with the same gene pair.	`NT_FUSION_SECONDARY` Values: • `FALSE` • `TRUE` If the key value pair is not included, then `NT_FUSION_SECONDARY` is assumed to be `FALSE`, indicating that a secondary isoform fusion is not found.

Exon tiling method

The exon tiling method is a partner agnostic fusion detection method that enables the discovery of novel fusion isoforms and breakpoints. In this method, the primers are designed to target a subset of exon-exon junctions of several driver genes (see Figure 3). Each driver gene in the test sample is analyzed individually. After the sequencing reads undergo normalization and baseline correction, the software measures the intragenic 3′ to 5′ expression ratio for each gene and compares the ratio to the baseline (normal sample). Genes that do not undergo a fusion event are expected to have a 3′ to 5′ expression ratio similar to the baseline (see Figure 4). Genes that undergo a fusion event typically have a 3′ to 5′ expression ratio greater than the baseline (see Figure 5). The imbalance score measures the magnitude of change in 3′ to 5′ expression ratio relative to the baseline. For each driver gene in which fusion was detected, the software also predicts the most likely position of fusion breakpoint. This allows for discovery of novel fusion breakpoints.

Imbalance score = Observed imbalance (test sample) ÷ Expected imbalance (normal sample)

For example, if the observed (test sample) 3′ to 5′ expression ratio is 3, although the expected 3′ to 5′ imbalance for a wild type transcript is 1.5, the imbalance score is 2. Typically, an imbalance score of ≥1.75–2 is indicative of a gene fusion event.

The significance of the expression imbalance is measured by the imbalance p-value. The p-value measures the significance of the imbalance at the predicted breakpoint compared to the negative control gene in the sample. Both, the p-value and the imbalance score are used to determine the occurrence of a fusion event.

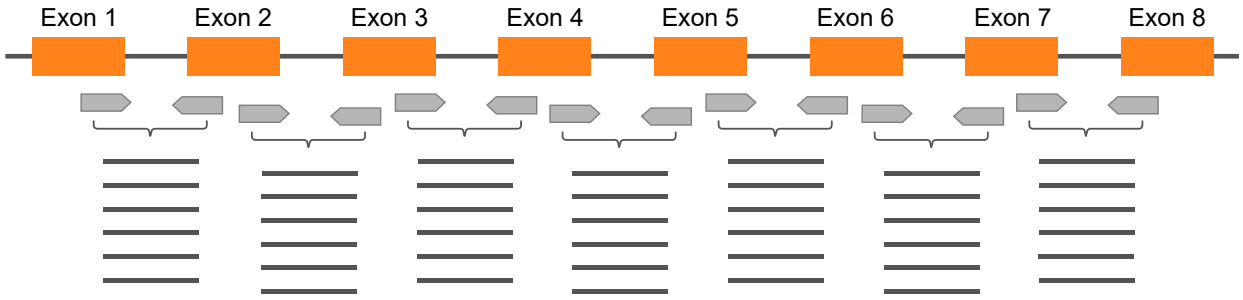


Figure 3 Representative primer design for an exon tiling assay

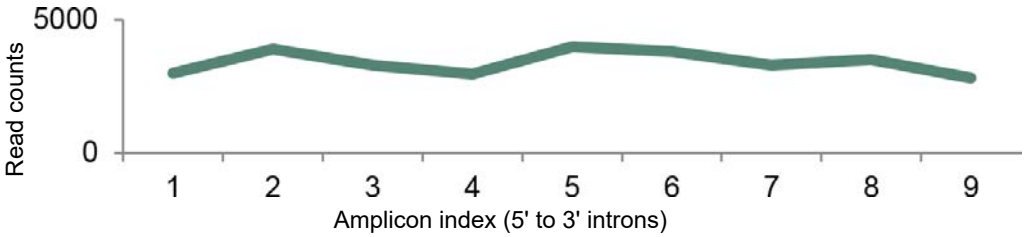


Figure 4 Example coverage profile for a sample with no fusion present

In this example, no fusion is present in the sample. The wild type transcript has uniform coverage of 3′ and 5′ introns.

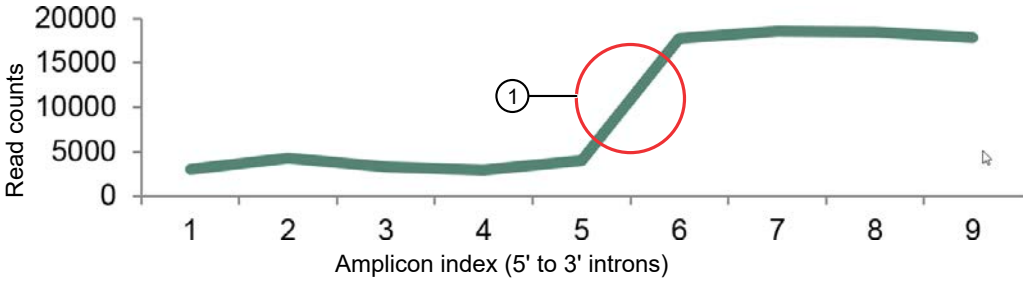


Figure 5 Example coverage profile for a sample with fusion present

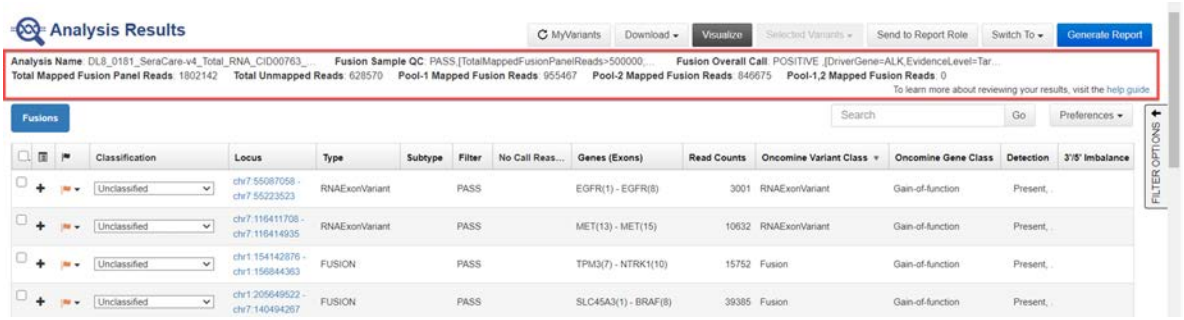
In this example, a mixture of wild type and fusion transcript is present in the sample. The presence of the fusion transcript accounts for the elevated expression of the 3′ gene region.

① Predicted fusion breakpoint

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



Metrics listed in the **Analysis Results** are displayed in the following table.

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** – although 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.

(continued)

Column	Description
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 supported by the read counts dependent on the thresholds set for detection.
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.
Read Counts Per Million	The ratio of target fusion read counts within the total mapped reads X 10^6
Variant ID	Shorthand identifier for the fusion variant, assay, or expression control. Each target in the Ion AmpliSeq™ panel has a unique variant identifier. The variant identifier can be from the Partner gene name, gene and exon number, COSMIC ID, NCBI GenBank ID, or non-targeted. For more information see the Ion Reporter™ Software help system.

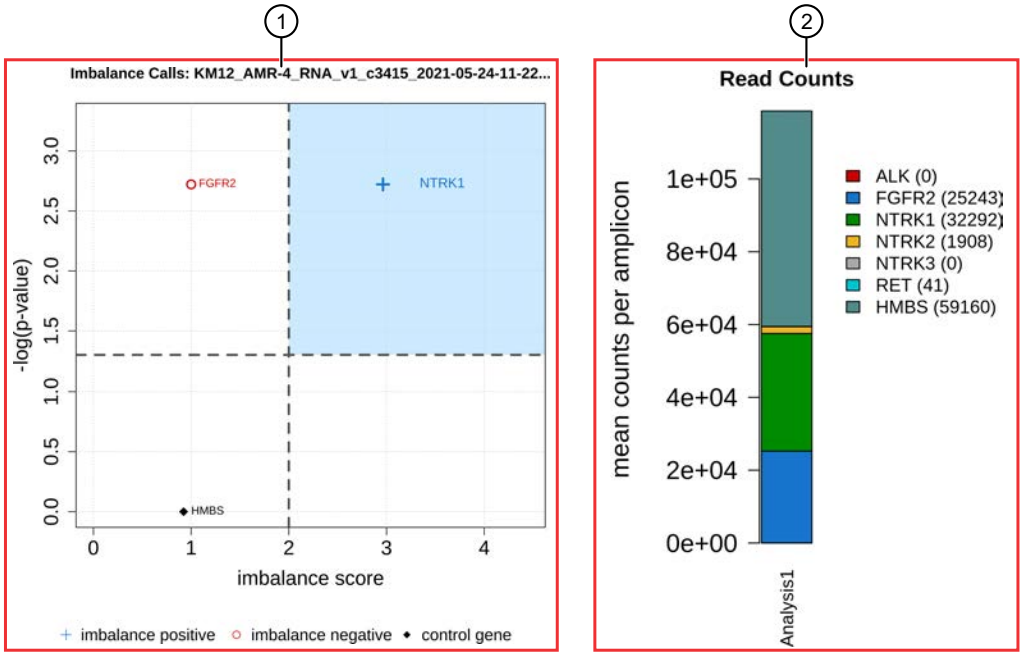
After you review, filter, and sort your Analysis Result, you can create a report (see "Generate a final report" on page 93), or download the analysis files (see "Download Ion Reporter™ Software annotation files" on page 94).

View RNA Exon Tile Fusion Imbalance

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

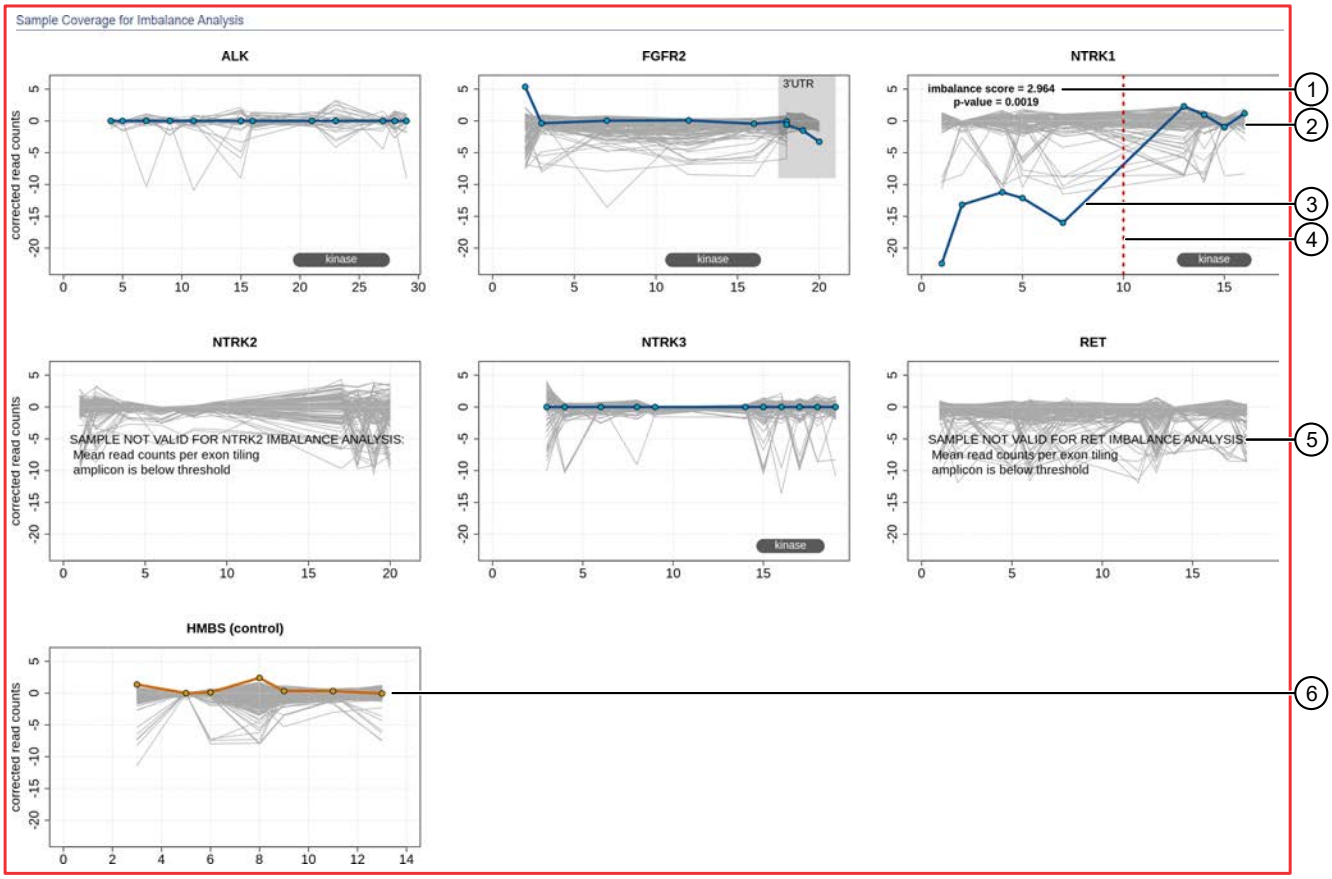
1. In the menu bar, click **Analyses** ▸ **Overview**.
2. In the **Analysis** column, click the hyperlinked name of the analysis of interest.
3. In the **Analysis Results** screen, click the **Variants** tab, then click **Fusions**.
   The **Fusions** table opens to display fusions results.
4. In the 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.

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.

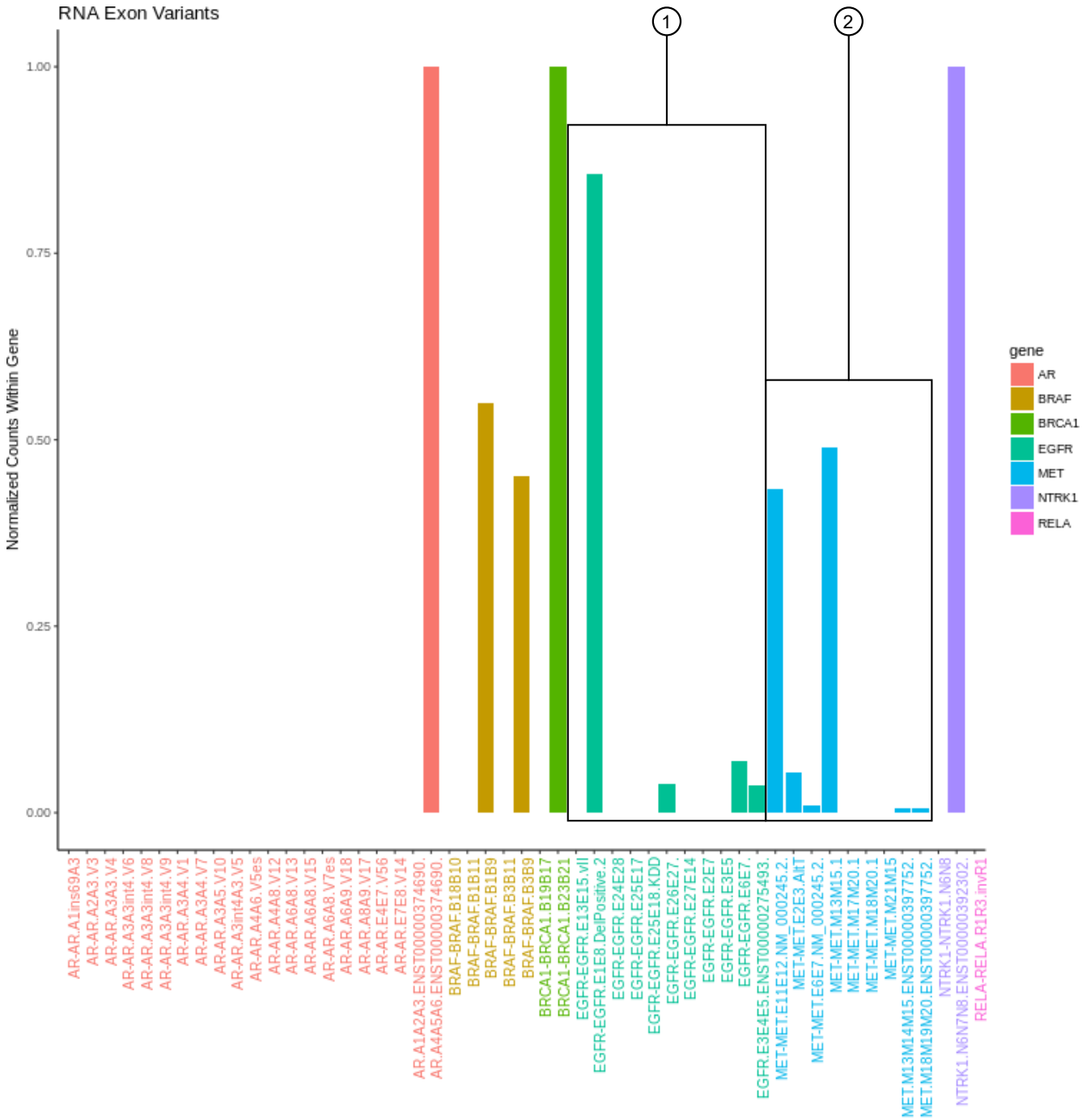
1. In the menu bar, click **Analyses** ▸ **Overview**.
2. In the **Analysis** column, click the hyperlinked name of the analysis of interest.

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).

Visualize tumor mutational burden analysis results

To visualize tumor mutational burden analysis results in Ion Reporter™ Software, the analysis workflow must be any DNA-single sample, or DNA and Fusions-single sample analysis workflow that has tumor mutational burden enabled.

1. Do one of the following to open tumor mutational burden analysis results:

Option	Description
Visualize analysis results from an individual sample or from multiple samples simultaneously from the **Analyses** table.	In the **Analyses** table, select an individual sample result row, or select the checkbox next to each sample result that you want to visualize simultaneously, then click **Visualize**. Alternatively, click **Actions** ▸ **Visualize**.
Visualize analysis results individually from the **Analysis Results** screen.	In the **Analyses** table, click a sample result hyperlink in the **Analysis** column to open the **Analysis Results**, then click **Visualize**.

   The **Tumor Mutational Burden (Mutations/Mb)** is displayed at the top of each section of sample results.

2. Click **Download Report** to download the **Tumor Mutational Burden** visualization report in PDF format, which includes the results with graphs and metrics.

3. Scroll down to view graphical representations of the analysis results and QC metrics, and download additional files.

   Note: Multiple sample results are listed sequentially.

4. In the lower right corner of the screen:

   - Click **Download Variant Details TSV** to download a tab-separated list of detected variants that contributed to the tumor mutational burden count. Open the file with compatible software, such as Microsoft™ Excel™.
   - Click **Download the TMB statistic.txt file** to download the tumor mutational burden output file. The file is named `<analysis name>_statistic.txt`. For example, `NCI-1395_c3495_2020-03-03-10-58-54-007_statistic.txt`.

QC metrics for tumor mutational burden

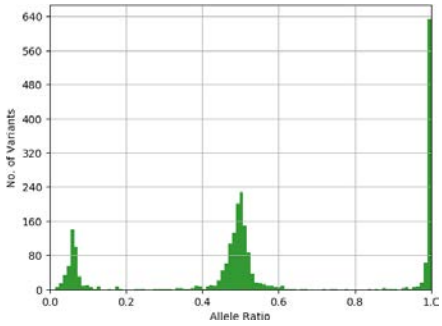
QC metrics for tumor mutational burden are listed at the top of **Analysis Visualization** in the **TMB** tab when you visualize analysis results with Ion Reporter™ Software.

QC metric	Definition
Average Coverage	The following formula is used to calculate average coverage: $\frac{\text{Total base coverage across the genomic positions defined by the assay BED file}}{\text{Number of bases in the DNA BED file}}$ If the average coverage is below 150, the analysis completes successfully but Tumor Mutational Burden is reported as `-1`. **Note:** This setting cannot be changed in Ion Reporter™ Software.
Number of bases used in calculating TMB	The number of bases used as a denominator for the tumor mutational burden calculation. Only bases with sufficient base coverage are used in the calculation, as defined in the workflow parameters. In the parameters, you can also select only the genomic regions covered by the panel to be used instead of all exonic regions. See the Ion Reporter™ Software help system for more information.
Number of variant calls	The number of somatic variants that are identified in the sample. This value is reported in the `statistic.txt` file as **Total Somatic Filtered Variants Count (numerator for TMB calculation)** and **Variant Count**. For more information, see Ion Reporter™ Software help system.
Deamination score	Deamination score was previously reported as the estimated SNP proportion consistent with deamination (mainly FFPE). The deamination score can be used to determine the quality of the FFPE sample. For more information on how to minimize the impact of high deamination on a tumor mutational burden score, see Ion Reporter™ Software help system. A deamination QC status of ***PASS/FAIL*** is also reported. The threshold for this QC can be adjusted in the analysis workflow parameter settings. For more information, see Ion Reporter™ Software help system.

Sample results

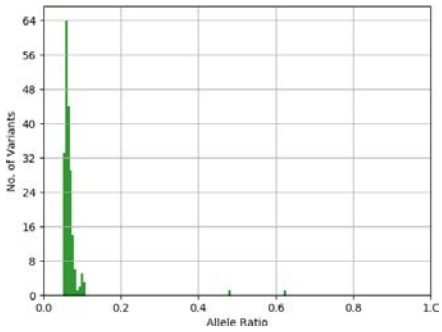
Germline and Somatic Variants

This histogram shows the frequency distribution of allele ratio for total called germline and somatic variants. Listed below the figure is the combined total of called germline and somatic variants. The value is reported in the `statistic.txt` file as **Variant count (Germline + somatic)**. For more information, see Ion Reporter™ Software help system.



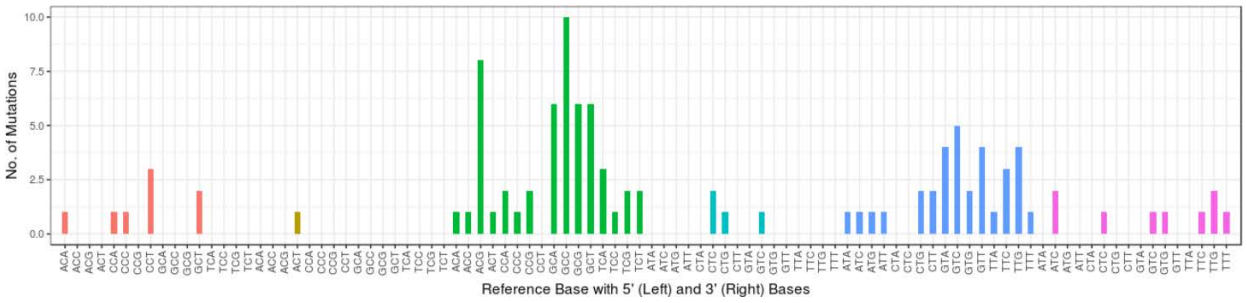
Only Somatic Variants

This histogram shows the frequency distribution of allele ratio for only somatic mutations as determined by the selected TMB filter chain. Listed below the figure are:



- The number of **Total Somatic Variants** , reported in the `statistic.txt` file as **Total Somatic Filtered Variants Count (numerator for TMB calculation)**.
- Of the **Total Somatic Variants** , the number of SNVs that are determined to be nonsynonymous (detrimental) and synonymous (non-detrimental) as annotated by Ion Reporter™ Software. Values are reported by different Ion Reporter™ annotation types in the `statistic.txt` file.
- The number of detected somatic variants found in the COSMIC database. The value is reported in the `statistic.txt` file as **COSMIC Annotated Somatic Variants**.

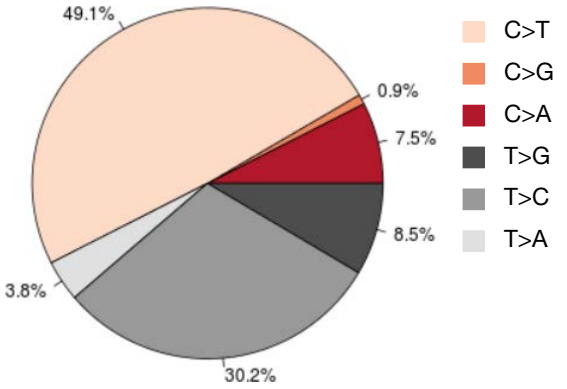
Substitution Type and Context of Somatic Mutations



Somatic mutations can be divided into 6 base substitution classes (that is, C>A, C>G, C>T, T>A, T>C, T>G) based on their substitution type. After incorporating information on the bases immediately 5′ and 3′ to each mutated base, 96 possible mutation types are in this classification. These 96 mutation types are represented on the x-axis, and variant frequency for mutation type on the y-axis. Bars for each substitution class are grouped and displayed with different color. A summary TXT file of these results is also available, see “Download Ion Reporter™ Software annotation files” on page 94.

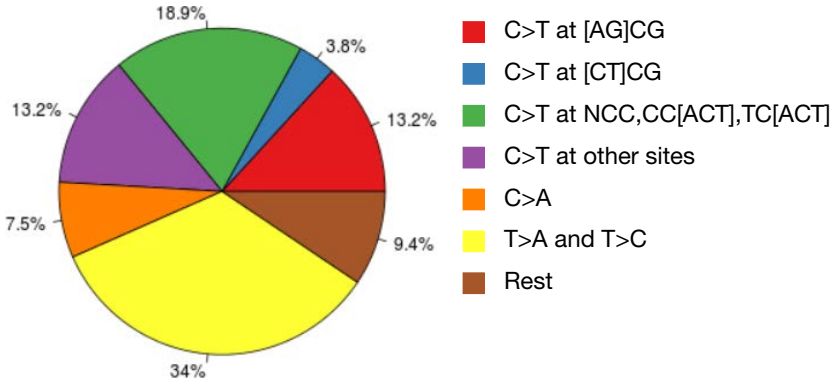
Substitution Type of Somatic Mutations

A pie chart dividing somatic mutations into 6 base substitution classes (that is, C>A, C>G, C>T, T>A, T>C, T>G) based on their substitution type.



Signature Pattern of Somatic Mutations

A pie chart dividing somatic mutations in groups consistent with specific mechanisms.



In the pie chart, a small fraction of multiple signature types can be observed in the sample. However, significant dominance of a single signature pattern often correlates to the respective tumor type. For example, 56.7% of the variants detected (sum of blue, green, and yellow) are an observed UV damage signature in this sample.

Note: Underlined bases represent the reference base being substituted (for example, CpG in the first bullet is same as to [ACGT]CG).

- High C>T at CpC, CpC, TpC, T>A, and T>C is consistent with UV damage. (Blue + Green + Yellow)
  For more information, see **https://www.nature.com/articles/nature22071**
- High C>T at CpG is consistent with spontaneous deamination of 5-methylcytosine. (Red + Blue).
  For more information, see **https://www.nature.com/articles/nature12477**
- High C>A is consistent with smoking damage.(Orange)
  For more information, see **https://science.sciencemag.org/content/354/6312/618.full**

- High C>T (site independent) is consistent with FFPE processing. (Green + Purple)
  For more information, see **https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4032349/**

Note: Samples of low quality that exhibit deamination (high C>T) can be analyzed if you increase the minimum allele frequency in an assay. For more information, see Ion Reporter™ Software help system.

Mutation Signature

Mutational Signature profiles

Cancer genomes are subject to diverse mutational processes that generate recognizable mutational signatures. Some processes are driven by defects in specific DNA repair pathways (for example, MMR) whereas others are characteristic of environmental mutagens (for example, ultra-violet light, tobacco smoke). These processes generate unique combinations of mutation types, a "Mutational Signature".

Somatic SNVs in the sample are filtered to remove germline mutations and are used to construct a normalised single base substitution (SBS) matrix, or mutational signature. The normalised sample mutational signature is then compared with 54 signatures from the COSMIC Mutational Signatures v3.1 and the cosine similarity is calculated. COSMIC signatures with cosine similarity score ≥0.7 to the sample signature are then analyzed with the deconstructSigs algorithm to identify which COSMIC Mutational Signatures are the best match.

View the Mutational Signature Prediction report

To view the mutational signature prediction report (`signature_identification_<analysis_name>_report.pdf`) you must first download the ZIP folder. For more information, see "Download Ion Reporter™ Software annotation files" on page 94. The folder contains the following files.

- PDF Report
- Profile of mutational signature of sample (Available only if a mutational signature match is found.)
- Profile of enriched COSMIC signature (One or more, available only if a mutational signature match is found.)
- deconstructSigs Weights Pie Chart (Available only if a mutational signature match is found.)
- Trinucleotide distribution of variants

1. Extract the downloaded ZIP folder to your hard drive.
2. Open the **signature_prediction** folder, then double-click the PDF Report file.

Mutational Signature Prediction report

File	Description
PDF Report	FileName format is “signature_identification_<analysis_name>_report.pdf”. The PDF report consists of 4 sections: **1.** Header • Report Name - "Mutational Signature Prediction Report" • Sample Name • Analysis Name (If a sample is re-analysed, the Analysis name will change) **2.** Summary • Table **a.** Name of enriched signature, from COSMIC **b.** Cosine similarity between sample mutational profile and COSMIC Signature **c.** Signature Description or Aetiology • COSMIC Signature Weights—pie chart denoting weights from deconstructSigs. This reflects the strength of the evidence for the match between the data and the Cosmic signature. **3.** Mutation Profiles **4.** Signature Specific Gene Mutations
Profile of Mutational signature of Sample	File name format is “<analysis_name>_normalized_sample_profile.png”. The image is present in the PDF report under “Mutation Profile” section.
Profile of COSMIC signature	File name format is “<analysis_name>_<cosmic_signature>.png”. The image is present in the PDF report under “Mutation Profile” section. One image is generated per enriched COSMIC signature. The output will not be generated if any enriched signature is not found.
deconstructSigs Weights Pie Chart	File name format is “<analysis_name>_signatures_pie.png”. The image is present in the PDF report under “Summary” section. The output will not be generated if any enriched signature is not found.
Trinucleotide distribution of Variants	File name format is “<analysis_name>_contextFile.txt”. It contains frequencies of mutations within each of the 96 trinucleotides.

Mutation Signature Prediction report example

① Header
② Summary
③ Mutation Profiles
④ Signature Specific Gene Mutations

View Oncomine™ BRCA analysis results

The provided Oncomine™ Comprehensive Assay Plus analysis workflows (Ion Reporter™ Software 5.16 or later) 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.

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[1]
	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
LongDel	—	A specific 40 bp deletion in BRCA1 (c.1176_1214del)

[1] Uses VCIB to determine BRCA1/BRCA2 gene deletion or duplication.

Visualize the BRCA report

Oncomine™ Comprehensive Assay Plus, DNA Ion Reporter™ Software analysis workflows enable the detection and visualization of whole exon and multiple exon deletions in BRCA1 and BRCA2 genes in somatic and germline samples with high sensitivity.

1. In the **Analysis Results** screen, click **Visualize**, then select the **BRCA** tab.

   The **Post-Corrected** view is shown by default. Click **Pre-Corrected** to view the uncorrected raw data.

   The **BRCA Report** is displayed in the **BRCA** tab. It shows a boxplot of read counts of each BRCA1 and BRCA2 exon that are normalized to the CNV baseline for the panel. In Ion Reporter™ Software 5.16 or later, 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.

   Note: For brevity, only genes from the HRR pathway are shown. Consequently, the figures cannot be used to interpret gain-of-function or loss-of-function from these genes.

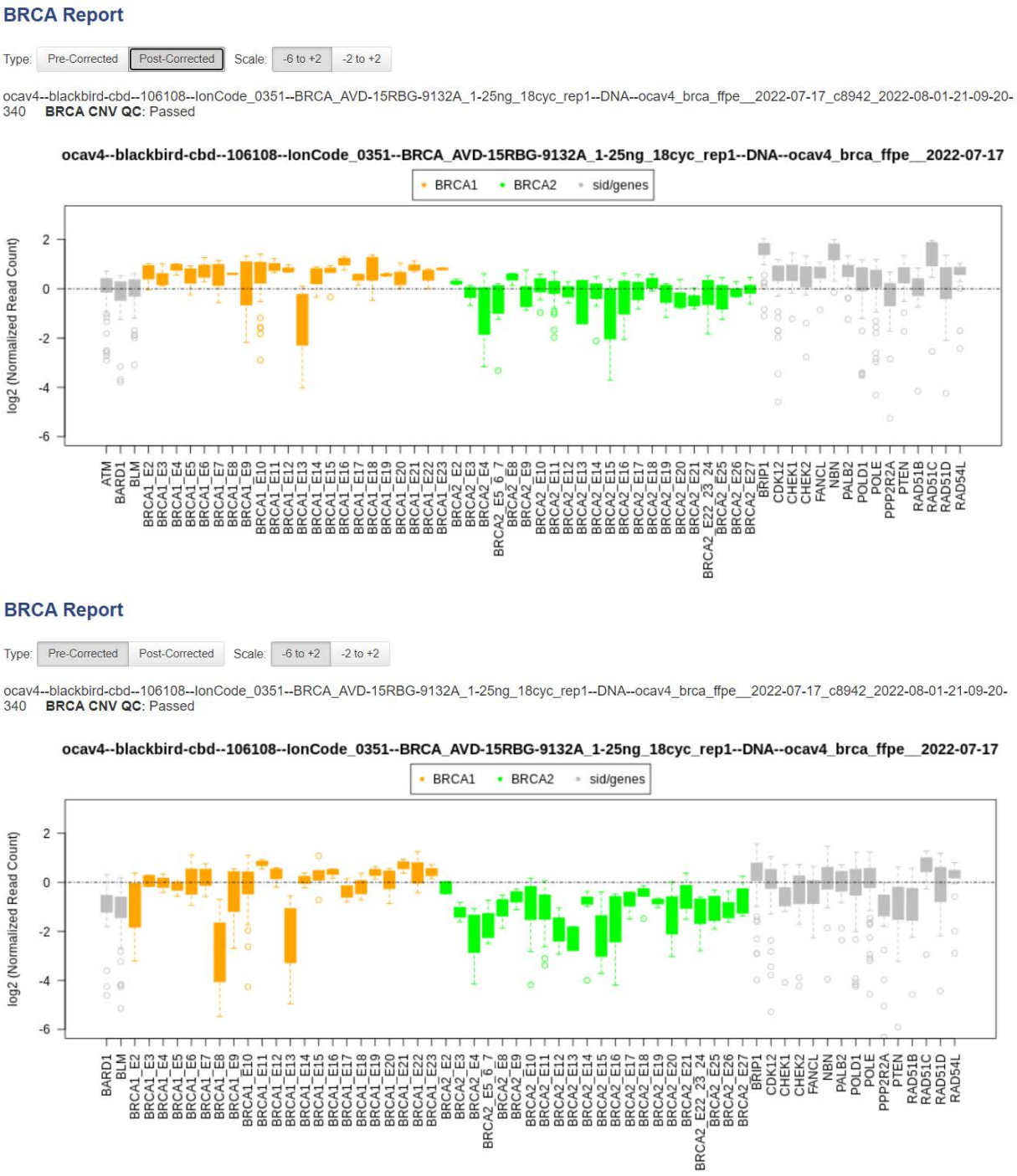
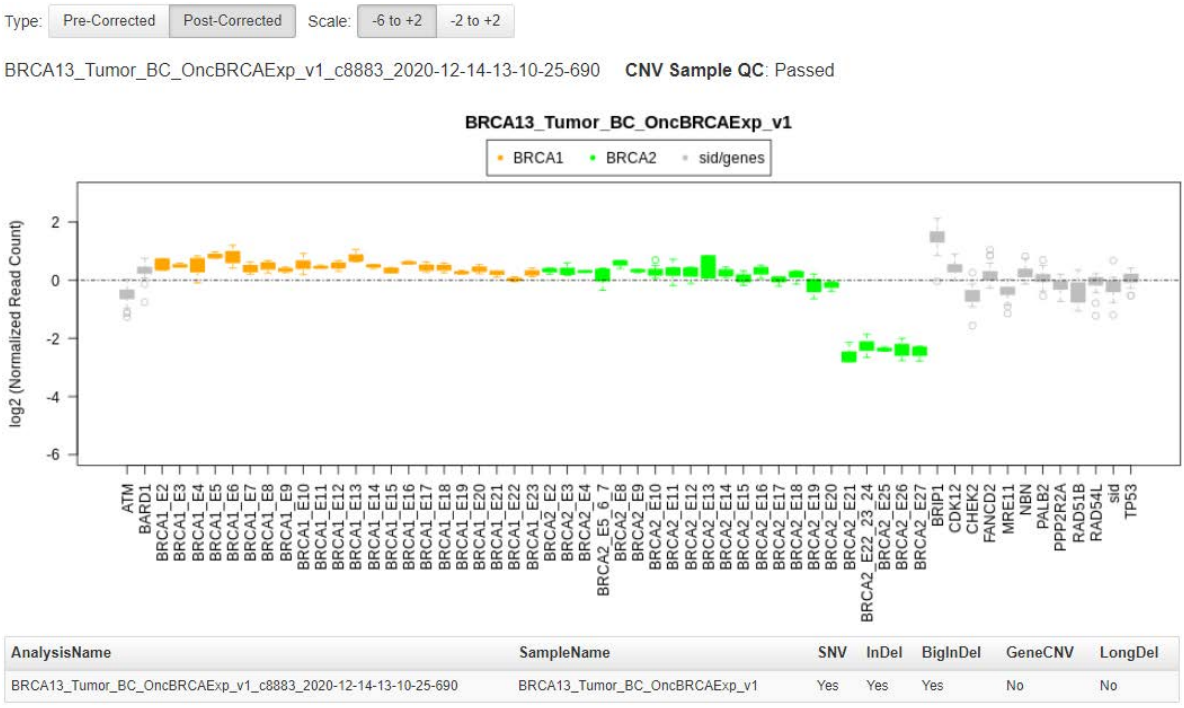


Figure 6 Normal sample, no BRCA whole-gene or exon deletion



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 7 Tumor sample with BRCA exon deletion

2. (Optional) To download the figures as PNG files, click **Download Results**.
   A ZIP file containing the PNG files for the graphs is downloaded.

3. Click the **IRGV & Generate Report** 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 higher than the selected **MAPD filter** value, no CNVs are shown 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.

4. 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.

Genomic segmentation analysis results

The Ion Reporter™ Oncomine™ Comprehensive Assay Plus, DNA analysis workflow uses heterozygous population SNPs covered by the assay to determine the ploidy levels of genomic segments.
The genome is divided into contiguous segments of similar ploidy levels using a circular binary segmentation (CBS) algorithm. Log odd ratios for variant allele frequency of observed population SNPs (by TVC) and copy-number (CN) ratios (by CNV pipeline) for each segment are calculated. Log odd ratio and CN ratios are then used to infer:

- Tumor cellularity percentage (percentage of tumor cells in the sample)
- Loss-of-heterozygosity (LOH) for each genomic segment. Segment level LOH events are intersected with targeted gene boundaries to determine LOH events in selected genes. Segment level LOH events are also aggregated to determine genomic (%LOH).
- Genomic Instability Metric (GIM)

To download the results, see “Download Ion Reporter™ Software annotation files” on page 94.

Visualization of genomic segmentation analysis, Allele Specific Copy Number plots

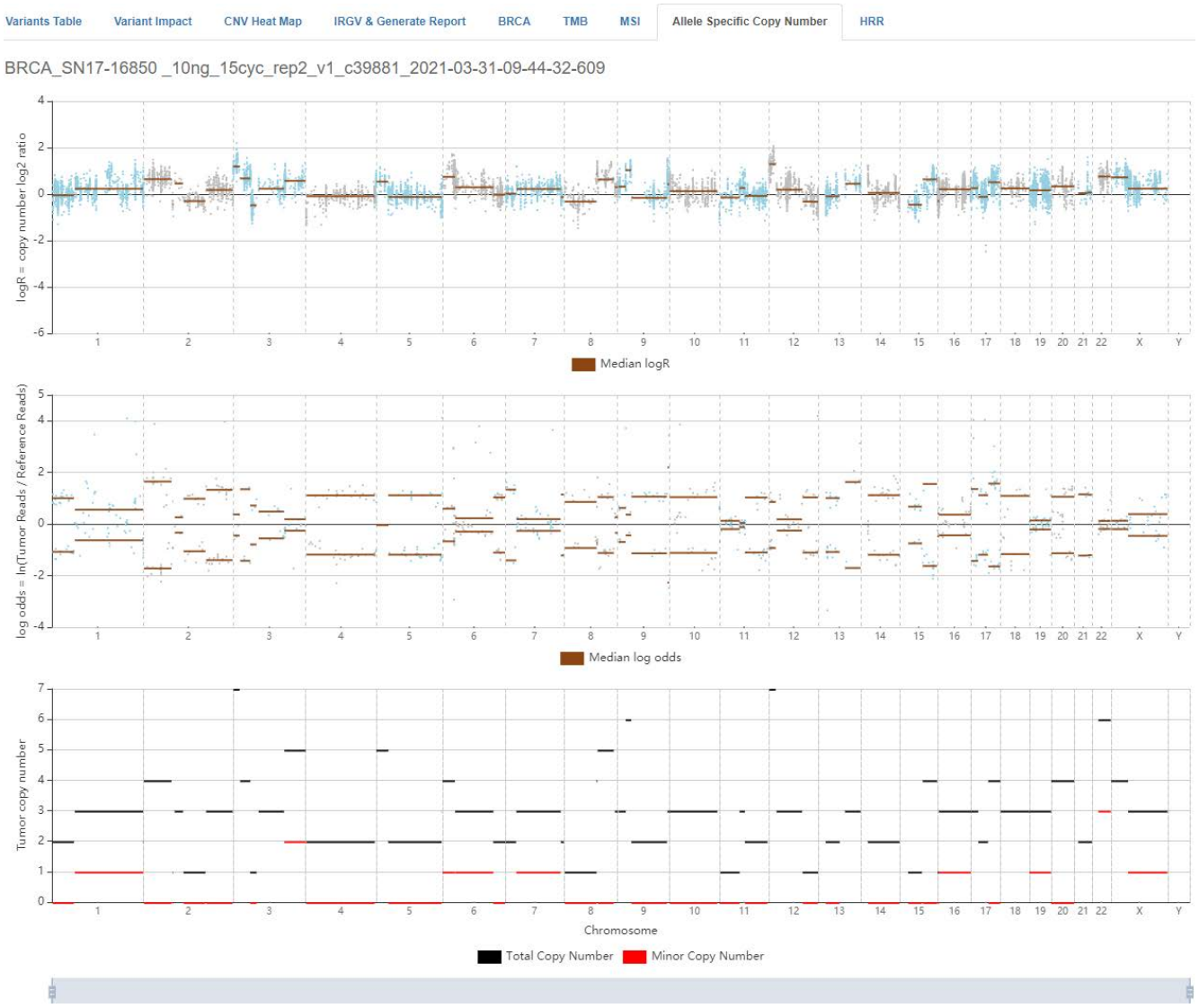
1. There are two ways to visualize analysis results in Ion Reporter™ Software:

Option	Description
Visualize analysis results from one or more analyses simultaneously from the **Analyses** table.	In the **Analyses** table, select a row for an analysis or select the checkbox next to one or more analyses that you want to visualize simultaneously, then click **Visualize**. Alternatively, click **Actions** ▸ **Visualize**.
Visualize analysis results individually from the **Analysis Results** screen.	In the **Analyses** table, click an analysis hyperlink in the **Analysis** column to open the **Analysis Results**, then click **Visualize**.

Note: The **Analysis Visualization** screen opens to the **TMB** tab by default.

2. Click the **Allele Specific Copy Number** tab.

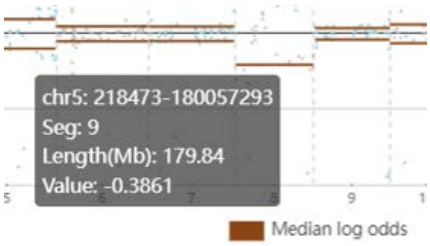
   The **Allele Specific Copy Number** tab displays plots showing copy number variation across the entire genome. If visualizing multiple analyses simultaneously, individual sample results are listed sequentially. This visualization shows identified genomic segments (shown as horizontal lines) using log2 ratios from the Copy Number Variation (CNV) analysis and log odds from the Torrent Variant Caller (TVC) analysis. Segments are identified using the heterozygous population SNPs (shown as dots in the upper two panels) targeted by assay amplicons.



Plot scale (x-axis)	Description
logR = copy number log2 ratio	Log2 ratios (top panel) of the copy-number estimates relative to the baseline copy-number as calculated by the CNV algorithm for each amplicon in the assay. Ratio representative Genome segmentation overlay (horizontal brown bars) shows segments with similar log2 ratios clustered together.
log odds = ln(Tumor Reads / Reference Reads)	Log odds (middle panel) for each heterozygous SNP in the assay. Log odds is calculated as the natural logarithm of the ratio of the sequencing reads with variant allele and reference allele that are obtained using Torrent Variant Caller (TVC). Genome segmentation overlay (horizontal brown bars) shows segments with similar log odds clustered together. Because the variant allele could be major or minor for any SNP and the corresponding log odds could be positive or negative, the long odds are displayed as segments that are mirror images around 0.
Tumor Copy Number	Bottom panel shows the total (black horizontal line) and minor (red horizontal line) copy-number estimates for each of the identified genomic segments (top two panels). Genomic segments where total CN ≥1 and minor CN = 0 are segments with LOH. There may exist genomic segments for which minor copy-number estimates can not be determined.

3. *(Optional)* Select one of the following actions.

   - To see the gene or gene segment information corresponding to the genomic loci, hover over a dot in the plot.



   - To save a PNG image of all three plots, click **Export Image** to save a PNG image of all three plots to a location of your choice.

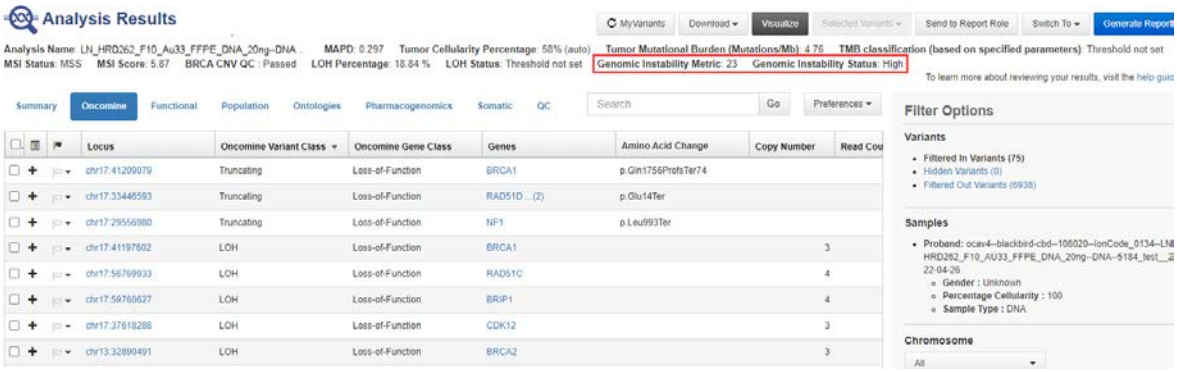
View Genomic Instability Metric (GIM) in analysis results

The Genomic Instability Metric (GIM) is a proprietary measurement that quantifies genomic scarring associated with Homologous Recombination Deficiency (HRD). Starting with Ion Reporter™ Software 5.20, Oncomine™ Comprehensive Assay Plus analyses report GIM.

GIM summarizes unbalanced copy number changes in autosomes that are determined using genomic segmentation, and the software generates a value between 0–100. Higher GIM values correlate with the observation of more genomic instability in the sample.

In addition to GIM, the Genomic Instability Status is also reported. Genomic Instability Status compares GIM to a threshold. Genomic Instability Status is reported as high if GIM is equal to or greater than the threshold or low otherwise. By default, the threshold is set at 16, which is based on ovarian cancer. If a threshold is not set, *Threshold not set* is reported. If GIM cannot be calculated, a value of *no call* is set.

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 analysis of interest to open **Analysis Results** screen.
   GIM and Genomic Instability Status are listed below the **Analysis Results** screen header (red box).



Visualize HRR analysis results

Cells use the Homologous Recombination Repair (HRR) pathway to repair double-strand breaks generated as by-products of cellular metabolism, DNA replication errors, or exogenous factors.

Homologous Recombination Deficiency (HRD) is the inability of cells to repair double-strand DNA breaks using the HRR pathway.

The HRR visualization table summarizes relevant loss-of-function variants to provide information and enable assessment about likely biallelic variants in that gene, for example, BRCA1 and BRCA2. In addition, the HRR visualization table displays the cause and consequences of HRD by displaying metrics for measuring HRD (genomic loss-of-heterozygosity in the sample (%LOH), Genomic Instability Metric (GIM) score, and Genomic Instability Status).

Gene list for gene-level LOH (Ion Reporter™ Software 5.20): ATM, BRCA1, BRCA2, BARD1, BRIP1, CDK12, CHEK1, CHEK2, FANCL, PALB2, RAD51B, RAD51C, RAD51D, and RAD54L.

1. Select one of the following options.

Option	Description
Visualize analysis results from an individual sample or from multiple samples simultaneously from the **Analyses** table.	In the **Analyses** table, select an individual sample result row, or select the checkbox next to each sample result that you want to visualize simultaneously, then click **Visualize**. Alternatively, click **Actions** ▸ **Visualize**.
Visualize analysis results individually from the **Analysis Results** screen.	In the **Analyses** table, click a sample result hyperlink in the **Analysis** column to open the **Analysis Results**, then click **Visualize**.

   The **Analysis Visualization** opens to the **TMB** tab by default.

2. Select the **HRR** tab to review a summary of the analysis results about the genes and genomic alterations in the HRR pathway. For example, you can view information about genomic LOH (sample %LOH), CNVs, SNVs, INDELs and the Genomic Instability Metric (GIM). You can also view any exon-level deletions and duplications that are associated with BRCA1 and BRCA2 genes.

   Note:
   - If variants meeting a category are not detected, **Not Detected** is reported.
   - You may see differences in variant calls reported in the Oncomine Variant tab or default variant table on the **Analyses** page and HRR summary table due to differences in the filter chain used and allele frequencies.
     - Variants in the HRR table are filtered using the TMB (Non-germline Mutations) filter chain. Variants in the default variants table are filtered using the Oncomine™ Extended filter chain (OEFC). OEFC retains pathogenic germline variants reported in ClinVar, whereas TMB (Non-germline Mutations) does not. As a result, the HRR summary table does not contain these variants.
     - Variants reported in HRR summary table have variant allele frequency ≥5%. As OEFC does not apply this criteria, all variant frequencies are reported in the default variant table.

3. Click **Download** ▸ **Current Results TSV** to download a tab-separated list of detected variants in the analysis results. Open the file with compatible software, such as Microsoft™ Excel™.

   For more information about downloading files, see “Download Ion Reporter™ Software annotation files” on page 94.

4. Click **Download** ▸ **Filtered Variants** or **Download** ▸ **All Variants** to download a ZIP folder of files that list the variants in the analysis results.

   The file `hrr_summary.tsv` is included in the downloaded results.

MSI analysis results

You can determine whether microsatallite instability (MSI) is present using Oncomine™ Comprehensive Assay Plus - w3.1 - DNA (or DNA and Fusions) Ion Reporter™ Software analysis workflows. In these analysis workflows parameters are applied that detect MSI markers, which can identify a form of genomic instability in the replication of repetitive DNA.

Note: MSI parameters in the software are not applied when you use the Oncomine™ Comprehensive Plus - w3.1 - Fusions - Single Sample workflow as MSI is a DNA variant.

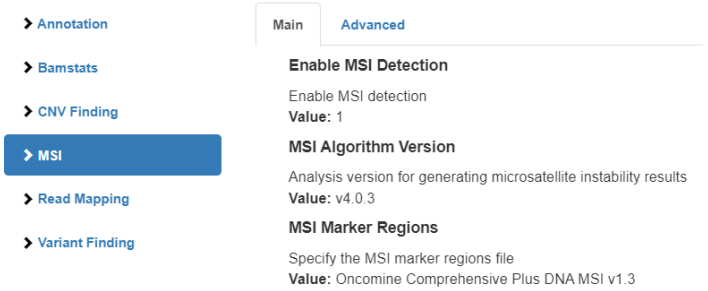
View MSI parameters

The parameters for MSI analysis are located in the Oncomine™ Comprehensive Plus - w3.1 - DNA - Single Sample workflow in Ion Reporter™ Software.

1. Under the **Workflows** tab, in the **Overview** screen, search for the workflow, then select it in the list.

2. In the **Details** pane to the right of the list, scroll down, then click **View** next to **Parameters**.

3. In the **Parameters** dialog box, select **MSI** from the left navigation bar.



MSI parameters

You can adjust MSI parameters to optimize your analysis results when you create or edit analysis workflows in Ion Reporter™ Software. MSI parameters are available only for some Oncomine™ analysis workflows. For a complete description of all MSI parameters see the Ion Reporter™ Software help system.

Parameter name	Description
Main tab	
Enable MSI Detection	When set to true, this parameter enables detection of MSI markers for the workflow. These markers can identify a form of genomic instability in the replication of repetitive DNA. MSI often occurs in tumor cells. It leads to the appearance of multiple alleles at microsatellite loci, which can be easily identified. This parameter is set to **True** by default in the Oncomine™ Comprehensive Assay Plus analysis workflows. If you do not want to include MSI results in an analysis, you can set to the parameter to **False**. Do not change this setting in other Oncomine™ analysis workflows.
MSI-High Threshold	The MSI score above which a sample is considered MSI-High. This score is reported in analysis results. [1,2]
MSS Threshold	The MSI score below which a sample will report MSS in analysis results.[1,2]

[1] If the MSI Score falls between the **MSI-High Threshold** and the **MSS Threshold**, a No Call is reported in the analysis results.

[2] The MSI-High and MSS thresholds are optimized based on the MSI baseline of the workflow. Do not change parameters from the default settings unless you understand how the change can affect your analysis.

Visualize MSI analysis results

1. There are two ways to visualize MSI analysis results in Ion Reporter™ Software:

Option	Description
Visualize MSI results from one or more analyses simultaneously from the **Analyses** table.	In the **Analyses** table, select a row for an analysis or select the checkbox next to one or more analyses that you want to visualize simultaneously, then click **Visualize**. Alternatively, click **Actions** ▸ **Visualize**.
Visualize analysis results individually from the **Analysis Results** screen.	In the **Analyses** table, click an analysis hyperlink in the **Analysis** column to open the **Analysis Results**, then click **Visualize**.

   The **Analysis Visualization** screen opens to the **TMB** tab.

2. Click the **MSI** tab.
   A table with **MSI Status**, **MSI Score**, **MSI Coverage**, and **MSI QC** is shown.

Item	Description
MSI Status	A sample is assigned an MSI status that is based on the MSI Score. The MSI status can be one of the following: • MSI-High—MSI score is greater than the threshold value set in the MSI-High Threshold parameter. • MSS—MSI score is less than the threshold value set in the MSS Threshold parameter. • No Call—MSI score is equal to or greater than the value entered in the **MSS Threshold** parameter AND less than or equal to the value entered in the **MSI-ThresholdHigh** parameter. • QCFail—Indicates determination of MSI status was not reliable due to the MSI baseline.
MSI Score	A sample-level MSI score that is calculated with individual MSI marker scores. The overall score is used to determine the MSI status of the sample.
MSI QC	Alert messages for quality control of the MSI analysis results. For more information, see “MSI QC Failure” on page 102. **MSI QC** is empty if the QC of MSI passes.
MSI Coverage	A combined sample-level coverage that is calculated with the individual MSI marker-level coverage.

3. Click **Download Results** to download a report of the MSI results. The report is downloaded to the folder that is used for downloads, depending on the browser settings.

View downloaded MSI results files

Extract the `MSIResults_<date>.zip` folder, then open the enclosed folder to access MSI results files.

The ZIP file of downloaded MSI results contains three files.

Filename	File type	Description
`Details.tsv`	Tab separated values (TSV)	File contains detailed information about each MSI marker in the assay. Details include Ion Reporter™ Software sample and analysis name, marker level coverage, MSI score, and MSI algorithm version.
`Summary.tsv`	Tab separated values (TSV)	File contains sample level summary of MSI results. This includes sample and analysis name in Ion Reporter™ Software, total coverage across all MSI markers, MSI score, MSI status, and MSI algorithm version.
ZIP folder containing `MSIQC.json`, `Calsats.json`, `Classify.json`, and `features.json`	JavaScript Object Notation (JSON)	• `MSIQC.json`—Provides the MSI status and MSI score results for the sample. File contains "flags" and warning messages associated with MSI status used by support personnel. See “Customer and technical support” on page 132. • `Calstats.json`—Several metrics used for selecting the MSI baseline. • `Classify.json`—Detailed output file from MSI algorithm. Contains information about different targets in addition to scores, status and QC messages • `features.json`—Contains marker and RMC features used for selecting the MSI baseline and calculating MSI marker scores.

Visualize MMR analysis results

The Mismatch Repair (MMR) pathway is critical to the repair of mismatch errors which typically occur during DNA replication. Mutations and loss of expression in MMR genes, known as defective MMR (dMMR), lead to microsatellite instability (MSI). In contrast, when MMR genes lack alterations, they are referred to as MMR proficient (pMMR). The biomarkers that are associated with MMR can indicate alterations in genes and genomes.

The MMR visualization table provides a summary of loss-of-function mutations in DNA mismatch repair pathway genes.

Variant types surveyed:

- SNVs : Missense and nonsense (germline filtered)
- INDELS: Frameshift and non-frameshift (germline filtered)
- Gene CNVs: Copy loss

Genes surveyed: MLH1, MLH3, MSH2, MSH3, MSH6, PMS2, POLD1, POLE, RPA1

1. Select one of the following options.

Option	Description
Visualize analysis results from an individual sample or from multiple samples simultaneously from the **Analyses** table.	In the **Analyses** table, select an individual sample result row, or select the checkbox next to each sample result that you want to visualize simultaneously, then click **Visualize**. Alternatively, click **Actions** ▸ **Visualize**.
Visualize analysis results individually from the **Analysis Results** screen.	In the **Analyses** table, click a sample result hyperlink in the **Analysis** column to open the **Analysis Results**, then click **Visualize**.

   The **Analysis Visualization** opens to the **TMB** tab by default.

2. Select the **MMR** tab to review a summary of the analysis results about alterations in the MMR pathway genes.

   Note: If variants meeting a category are not detected, **Not Detected** is reported.

3. Click **Download** ▸ **Current Results TSV** to download a tab-separated list of detected variants in the analysis results. Open the file with compatible software, such as Microsoft™ Excel™.

   For more information about downloading files, see “Download Ion Reporter™ Software annotation files” on page 94.

4. Click **Download** ▸ **Filtered Variants** or **Download** ▸ **All Variants** to download a ZIP folder of files that list the variants in the analysis results.

   The file `mmr_summary.tsv` is included in the downloaded results.

Generate a final report

After you have reviewed, filtered, and sorted your analysis results, you can download a final report. The procedure described here includes creating and formatting a final 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 final report can be rearranged, deleted, or edited.

2. Hover the cursor over the various sections and icons to view instructional text to help you format your final 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 final report template for future use with other sample results.

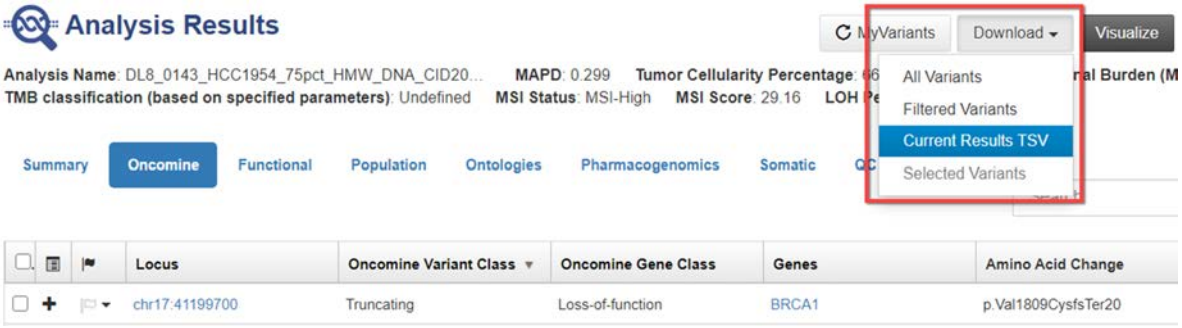
5. Click **Next**, a live preview of your final report is displayed.

6. Click **Lock & Publish** to generate the final report.
7. Click **Download**.

Download Ion Reporter™ Software annotation 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 in Ion Reporter™ Software.

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 folder named <analysis name>_All.zip.

3. Extract the contents of the compressed folder.

   The extracted folder contains the following files.

Folder	Contents of folder
CNV_VCIB	• `amplicon_data.txt` Open the file with a text editor application. • `cn_results.png`, an image file that represents the CNV amplicons in the X scale and log2 ratio in Y scale.
QC	Contains a PDF of the QC report, and a folder containing coverage statistics files.

(continued)

Folder	Contents of folder
Variants	Contains VCF and TSV output files. You can open TSV files in Microsoft™ Excel™ and VCF files in any text editor. • Intermediate and Oncomine™-annotated .VCF files • TSV files that contain Oncomine™-filtered and all somatic variants
Results	If the Tumor Mutational Burden parameter is enabled a Results folder is generated. The Results folder contains: • A PDF report of the tumor mutational burden results • TSV files that contain post-filter and somatic variants • Image files that graphically represent the results • TXT files that contain summaries of the TMB output • TXT files of the base changes
MSI	Contains summary and detailed marker-level files with data about microsatellite markers. These markers can identify a form of genomic instability in the replication of repetitive DNA. • `Summary.tsv`—Provides a summary of results for microsatellite markers, as shown in the **Analysis Results** table. • `Details.tsv`—Provides marker-level information, such as individual marker MSI scores, and read coverage for individual microsatellite markers. • ZIP folder—Contains the following files. – `MSIQC.json`—Provides the MSI status and MSI score results for the sample. File contains "flags" and warning messages associated with MSI status used by support personnel. See “Customer and technical support” on page 132. – `Calstats.json`—Several metrics used for selecting the MSI baseline. – `Classify.json`—Detailed output file from MSI algorithm. Contains information about different targets in addition to scores, status and QC messages – `features.json`—Contains marker and RMC features used for selecting the MSI baseline and calculating MSI marker scores.
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.
CnvActor	• `Gene_deletions.xls`, a file that contains information about whole gene deletions and amplicon coverage that are found in BRCA analyses. • TumorFraction folder – `tumor_fraction.json`—Several key metrics, intermediate output file location, filter and flags associated with the genome segmentation output. – `tumor_fraction_plot.png`—Genome segmentation plot (same as displayed under **Allele Specific Copy Number** tab, see page 85)
HRR_Results	Contains a TSV file with a summary of the HRR results.
MMR_Results	Contains a TSV file with a summary of the MMR results.

(continued)

Folder	Contents of folder
signature_prediction	The signature_prediction folder contains: • A PDF report of the mutational signature prediction results • Image files that graphically represent the results (Only available if a mutational signature match is found.) • A TXT file of the trinucleotide distribution of Variants
BRCAResults.zip	• BRCA1 and BRCA2 box plots (PNG files) that are same as what's displayed under the 'BRCA' visualization tab on UI • TSV file summarizing different variant types detected in BRCA1 and BRCA2 genes. • A folder, 'CnVActor-00' with a text file containing the BRCA CNV QC.

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

Requirements for variant annotation in Ion Reporter™ Software

The following table summarizes the requirements that must be met for the Oncomine™ Variant Annotator to annotate variants in Ion Reporter™ Software for the Oncomine™ Comprehensive Assay Plus.

For each variant type in this table, an annotation is generated only if all conditions in the corresponding **Annotation Criteria** column are satisfied.

Note: You can find all relevant annotation criteria in VCF files.

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"
Copy number exon duplication	**Loss-of-Function**	**ExonDuplication**	• SVTYPE = "CNV" • FILTER = "GAIN" • SUBTYPE = "BigDup"

(continued)

Variant type	Oncomine™ Gene class	Oncomine™ Variant class	Annotation criteria
Loss of Heterozygosity	**Loss-of-Function**	**LOH**	• SVTYPE = "LOH" • LOH = "1" • FILTER = "GAIN" or "LOSS" or "PASS"
Gene fusion	**Gain-of-Function**	**Fusion**	• SVTYPE = "Fusion" • FILTER = "PASS" • Is a targeted fusion isoform
Non-targeted fusion	**Gain-of-Function**	**NonTargetedFusion**	• SVTYPE = "Fusion" • FILTER = "PASS" • Non-Targeted field is present • NT_FUSION_IN_FRAME is not FALSE • NT_FUSION_DRIVER_INVOLVED is not FALSE • NT_FUSION_SECONDARY is not TRUE
RNA exon variant	**Gain-of-Function**	**RNAExonVariant**	• SVTYPE = "RNAExonVariant" or "Fusion" • FILTER = "PASS" • Is a targeted RNA exon variant
Expression imbalance	**Gain-of-Function**	**ExpressionImbalance**	• SVTYPE = "RNAExonTiles" • FILTER = "PASS" • Record meets Targeted Isoforms Detected Requirement
Long deletion	**Loss-of-Function**	**LongDel**	• Positive mutation call • SVTYPE = "LongDel"
Loss-of-function truncating de novo mutation	**Loss-of-Function**	**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**	**Hotspot**	• Positive mutation call • Functional impact is missense • Transcript and codon position occur in predefined missense hotspot list

(continued)

Variant type	Oncomine™ Gene class	Oncomine™ Variant class	Annotation criteria
In-frame hotspot mutation	**Gain-of-Function** **Loss-of-Function**	**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**	**Hotspot**	• Positive mutation call • Transcript, location, and exon occur in predefined splice site hotspot list
Intronic hotspot mutation	**Gain-of-Function** **Loss-of-Function**	**Hotspot**	• Positive mutation call • Transcript, location, and coding syntax occur in predefined intronic hotspot list
Promoter hotspot mutation	**Gain-of-Function** **Loss-of-Function**	**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**	**Hotspot**	• Positive mutation call • Transcript and coding syntax occur in MNV hotspot list
EGFR exon 19 deletion[1]	**Gain-of-Function**	**EGFRExon19Deletion**	• Positive mutation call • Functional impact is nonframeshift deletion, nonframeshift block substitution • Deletion impacts codons 744–761 of EGFR

(continued)

Variant type	Oncomine™ Gene class	Oncomine™ Variant class	Annotation criteria
EGFR exon 20 insertion[1]	**Gain-of-Function**	**EGFRExon20Insertion**	• Positive mutation call • Functional impact is nonframeshift insertion or nonframeshift block substitution • Insertion impacts codons 762–775 of EGFR or variant is in EGFR exon 20 insertion confirmed list
ERBB2 exon 20 insertion[1]	**Gain-of-Function**	**ERBB2Exon20Insertion**	• Positive mutation call • Functional impact is nonframeshift insertion, nonframeshift block substitution • Insertion impacts codons 770–783 of ERBB2
JAK2 exon 12 indel[1]	**Loss-of-Function**	**JAK2Exon12Indel**	• Positive mutation call • Functional impact is nonframeshift deletion, nonframeshift insertion, or nonframeshift block substitution • Indel impacts codons 536–547 of JAK2
KIT exon 11 deletion[1]	**Gain-of-Function**	**KITExon11Deletion**	• Positive mutation call • Functional impact is nonframeshift deletion, or nonframeshift block substitution • Deletion impacts codons 550–580 of KIT • Alternatively, mutation occurs in splice site flanking the 5' end of exon 11
KIT exon 11 insertion[1]	**Gain-of-Function**	**KITExon11Insertion**	• Positive mutation call • Functional impact is nonframeshift insertion, or nonframeshift block substitution • Insertion impacts codons 550–580 of KIT
MET exon 14 skipping[1]	**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
PDGFRA exon 18 deletion[1]	**Gain-of-Function**	**PDGFRAExon18Deletion**	• Positive mutation call • Functional impact is nonframeshift deletion, or nonframeshift block substitution • Deletion impacts codons 841–847 of PDGFRA

[1] For more information, see Ion Reporter™ Software help system.

A Tips and troubleshooting

Tips

- Arrange samples in alternating columns on the plate for easier pipetting with multichannel pipettes during purification with the DynaMag™–96 Side Magnet.
- Plate seals can be firmly applied using the applicator in the MicroAmp™ Adhesive Film Applicator. Plate seals can be removed with less effort when hot. Try removing seals right after taking the plate out of the thermal cycler.
- Use IonCode™ Barcode Adapters to avoid handling and diluting adapters. Alternatively, combine and dilute Ion Xpress™ Barcode Adapters in large batches, then carefully aliquot into 96-well plates.
- If library yield is below 50 pM, libraries can still be sequenced by adding a proportionally larger volume to a combined library or template preparation.
- When amplifying multiple samples in a single PCR plate, ensure that the input across the samples is roughly equivalent so that the selected cycle number is optimal for all the samples in the run.

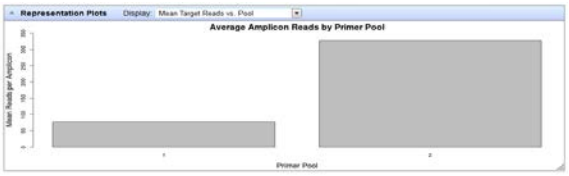
Troubleshooting

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. Requantify sample RNA with a Qubit™ Fluorometer.
	Sample DNA or RNA quality was low.	Add more DNA or RNA or increase target amplification cycles.
	PCR, digestion, or ligation was inefficient.	Ensure proper dispensing and mixing of viscous components at each step.
	Residual ethanol in the sample DNA or RNA inhibited target amplification.	Incubate uncapped tube in hood for 1 hour.
		Speed-vac tube at room temperature for 5 minutes.

Observation	Possible cause	Recommended action
Library concentration is low–general **Details:** (Library concentration is NOT indicative of quality.) *(continued)*	Residual ethanol from AMPure™ purification inhibited library amplification.	Carefully remove all drops of ethanol before library amplification, then centrifuge plate, if needed.
	AMPure™ XP beads were over-dried.	Do not dry the AMPure™ XP beads more than 5 minutes.
	FFPE RNA was not heat treated before reverse transcription.	Heat FFPE RNA at 80°C for 10 minutes, then cool to room temperature before reverse transcribing.
Library concentration 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.

Low amplicon uniformity (DNA only)

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.
	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.
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).

Other

Observation	Possible cause	Recommended action
The number of on-target reads is lower than expected	Unknown.	Increase the number of target amplification cycles by 2.
	Sample ID Panel targets were counted as off-target reads.	Add back the on-target reads from the Sample ID Panel.
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.
MSI QC Failure **Details:** Error message: "Not enough markers with coverage X"	MSI marker(s) had insufficient coverage to reliably calculate MSI. This is common in NTC samples.	If the sample is not an NTC, review the previous “Troubleshooting” topics to ensure sufficient coverage. For further assistance, contact support (see “Customer and technical support” on page 132).
MSI QC Failure **Details:** Error message: "RMC Calibration Failure"	Insufficient RMC read coverage for calibration. RMC reagent may have been omitted during library preparation.	Remake library including RMC in the target amplification reaction, then sequence the new library.
MSI QC Failure **Details:** Error message: "Severe imbalance in A and T scores"	Significant discrepancy in the score reported from A homopolymer vs the score reported by cognate T homopolymer. Sequencing conditions are impacting one nucleotide in a way that is significantly different from the other nucleotide.	Check that: • Instrument operating conditons are within the acceptable range. • Reagents in use have not expired. Re-sequence the library that failed after addressing all instrument or expired reagent issues.
Analysis failure due to high ploidy	The sample has high ploidy (≥11).	Reanalyze the sample using a custom workflow with CNV calling disabled. See “Copy-edit an analysis workflow to turn off CNV analysis” on page 111.

B Supplemental information

Update Oncomine™ Comprehensive Assay Plus templates in Torrent Suite™ Software

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™ Comprehensive Assay Plus, then in that row click **Update**.

The software update begins automatically and when finished shows a status of **Complete** in the **Update** column.

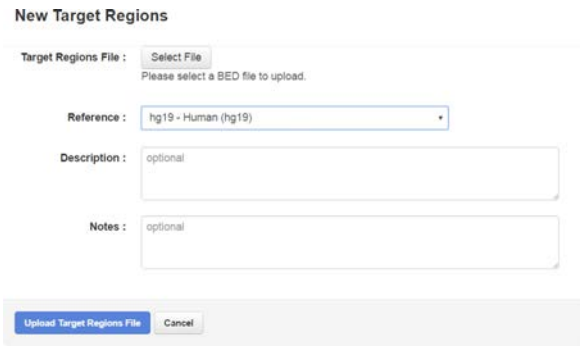
Install Oncomine™ Comprehensive Assay Plus analysis workflows in Ion Reporter™ Software

To install or update the Oncomine™ Comprehensive Assay Plus analysis workflows in Ion Reporter™ Software, contact your service representative to schedule an update.

Download and install BED files

Contact your service representative to obtain the latest versions of Oncomine™ Comprehensive Assay Plus BED files.

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: `.designed.bed`.



   d. Click **Open**, then click **Upload Target Regions File**.
5. Upload the hotspots BED file.
   a. In the left navigation menu, click **Hotspots**, then click the **Add Hotspots** button.
   b. Select **hg19 - Homo sapiens** from the **Reference** dropdown list.
   c. Click **Select File**, then navigate to and select the hotspots file, which has the following extension: `.hotspots.bed .`
   d. Click **Open**, then click **Upload Hotspots File**.

The installed BED files appear in the dropdown lists in the Ion Reporter™ Software.

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 a server type. [1]
Display Name	Enter a meaningful name of your choice. This name is used in the Planned Run template wizard and is shown to 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 this value.

5. The "Default Account" is the account that is configured by default in Planned Run templates and Planned Runs. 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.

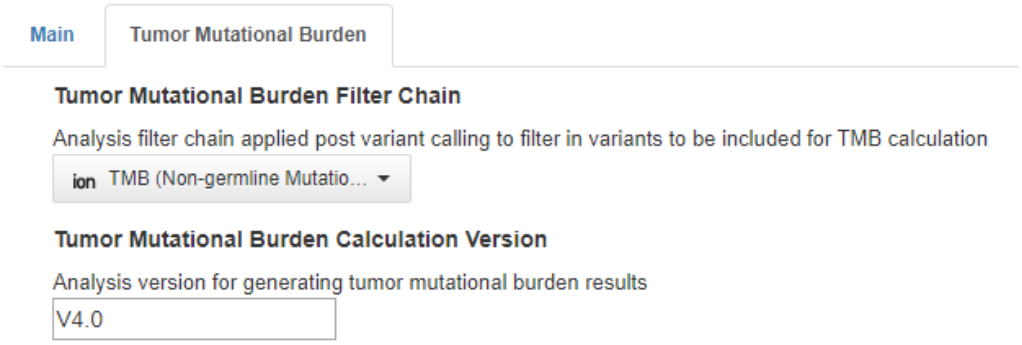
6. Click **Get Versions**, select **Ion Reporter 5.20 or later**, then click ✓**Add**.

Set TMB Classification parameters

Before the Ion Reporter™ Software analysis workflow can determine the TMB classification the threshold parameters must be set in the analysis workflow. Default threshold values (0) result in a value of **Undefined**.

1. Under the **Workflows** tab, in the **Overview** screen, search for the workflow, then select it in the list.
2. In the **Details** pane to the right of the list, click ⚙ ▸ **Copy** (to create a new analysis workflow) or **Edit** to modify the existing analysis workflow.
3. Advance to the **Parameters** step in the workflow bar.

4. Click the **Tumor Mutation Burden** tab.
   The **Tumor Muational Burden Filter Chain** and the **Tumor Mutational Burden Calculation Version** are displayed.



5. Scroll to **TMB-Low Threshold**, then enter threshold values for the following parameters.

Item	Description
TMB-Low Threshold	Tumor Mutational Burden (mut/mb) threshold below which a sample is defined TMB-Low.
TMB-High Threshold	Tumor Mutational Burden (mut/mb) threshold above which a sample is defined TMB-High.

6. Click **Next** to advance to the **Confirm** step, then click **Save Workflow**.

Reduce the impact of deamination in low-quality FFPEs

Samples of low quality that exhibit deamination can be analyzed if you increase the minimum allele frequency in an assay. For example, a minimum allele frequency of 10% for the tumor mutational burden (TMB) calculation can reduce the impact of deamination on the reported TMB value. It is important to consider the tumor content of a given sample when you increase the minimum allele frequency of the TMB calculation.

- Samples with low tumor content can have many true somatic mutations that are excluded from the tumor mutational burden calculation when a higher minimum allele frequency filter is included in the assay.
- The tumor mutational burden values cannot be reliable if you adjust the minimum allele frequency parameters for samples with a high estimated SNP proportion consistent with deamination, (primarily an FFPE number) or a high deamination score, (for example >100).
- The mean depth and uniformity of the sample as determined by the coverageAnalysis plugin can also indicate sample quality. For more information, see Ion Reporter™ Software help system.

IMPORTANT! Use the default parameter settings unless you are an advanced user.

Copy-edit an analysis workflow to reduce the impact of deamination

Low quality FFPE samples can impact the tumor mutational burden (TMB) calculation. To reduce the effect of deamination on the reported TMB value in low quality FFPE samples copy-edit an analysis workflwo as follows.

1. Sign in to the Ion Reporter™ Software.
2. In the **Workflows** tab, select the checkbox in the row of the analysis workflow to be copied, then click **(Actions)** ▸ **Copy**.
3. Click through each step in the workflow bar untilyou are in the **Parameters** workflow step.
4. In the **Parameters** step, select **Annotations**, then click the **Tumor Mutation Burden** tab.
5. Increase the **TMB Variant Minimum Allele Frequency** parameter (default value = 0.05 (i.e., 5% allele frequency)) up to 0.1 (10% allele frequency).
6. Click **Next**to advance to the **Confirm** step, enter a **Workflow Name** and **Description** (*optional*), then click **Save Workflow**.

The customized analysis workflow is now available in the Workflows screen.

Ion Torrent™ Class-Based Variants

Ion Torrent™ Class-Based Variants are a set of logic-based rules that Ion Reporter™ Software uses to computationally identify and annotate novel mutations in important genomic variant classes. The annotation logic is based on scientific literature evidence. Details about the logic follow.

EGFR exon 19 deletion

- **Background:** Epidermal growth factor receptor (EGFR) is a transmembrane receptor protein made up of 1210 amino acids and located on chromosome 7p11.2. EGFR has 31 exons— exons 2-4 and 8-12 encode the ligand binding domains, exons 5-7 and 13-16 encode the cysteine rich domain, exons 18–24 encode the tyrosine kinase domain (TKD), and exons 25-28 encode the autophosphorylation region [PMID: 17318210]. Mutations in EGFR often target the TKD which make up the ATP-binding pocket of the enzyme [PMID: 17318210]. Exon 19 deletions are indels that range between 15-18 bp in length and usually occur within codons 746-756 [PMID: 23768755, PMID: 22190593, PMID: 26933124, PMID: 24163741]. In non-small cell lung cancer (NSCLC), EGFR exon 19 deletions, located within codons 746-750, represent 45-50% of somatic mutations [PMID: 26933124, PMID: 24163741]. Exon 19 deletions result in constitutive activation of the receptor tyrosine kinase and hyperactivation of downstream signaling pathways [PMID: 29455648].
- **CBV Criteria:** The boundaries defined to capture EGFR exon 19 deletions as a class-based variant start at codon 744 and end at codon 761, which encompass common and rare indels [PMID: 30473385].

EGFR exon 20 insertion

- **Background:** Epidermal growth factor receptor (EGFR) is a transmembrane receptor protein made up of 1210 amino acids located on chromosome 7p11.2. EGFR has 31 exons— exons 2-4 and 8-12 encode the ligand binding domains, exons 5-7 and 13-16 encode the cysteine rich domain, exons 18–24 encode the tyrosine kinase domain (TKD), and exons 25-28 encode the autophosphorylation region [PMID: 17318210]. Mutations in EGFR often target the TKD which make up the ATP-binding pocket of the enzyme [PMID: 17318210]. In-frame insertions within exon 20 of EGFR are the third most common type of mutation found in NSCLC, representing 4-12% of all EGFR mutations in NSCLC [PMID: 31208370]. EGFR exon 20 encompasses codons 762 to 823. Exon 20 insertions commonly involve codons 762 to 774 wherein codons 762-766 make up the c-helix and codons 767-774 make up the activation loop [PMID: 31208370, PMID: 30854234]. EGFR exon 20 insertions do not alter the receptor binding affinity; however, it is suggested that the location of exon 20 insertions stabilizes its active confirmation [PMID: 30854234, PMID: 27843613].
- **CBV Criteria:** The boundaries defined to capture EGFR exon 20 insertions as a class-based variant start at codon 762 and end at codon 775. Additionally, known EGFR exon 20 insertions p.A763_Y764insFQEA and p.A763_Y764insLQEA, represented as c.2284–6_2284–5insTCCAGGAAGCCT and c.2284–9_2284–8insCCCTCCAGGAAG due to left alignment into the intronic region, are included in this rule.

ERBB2 exon 20 insertion

- **Background:** Erb-b2 receptor tyrosine kinase 2 (ERBB2) is a transmembrane glycoprotein located on chromosome 17q12 [PMID: 25276427]. ERBB2 (also known as HER2) has 27 exons— exons 2-4 and 9-12 encode the extracellular receptor L domains, exons 5-8 encode the furin like domain, exons 13-16 encode the growth factor receptor domain IV, exon 17 encodes the transmembrane domain, and exons 18–24 encode the tyrosine kinase domain (TKD) [PMID: 22761469, PMID: 29420467]. In lung cancer, the most recurrent ERBB2 activating mutations include in-frame exon 20 insertions [PMID: 30425522]. ERBB2 exon 20 involves codons 770 to 831 and majority of exon 20 insertions occur between codons 775 and 781 within the kinase domain [PMID: 29686424, PMID:22761469]. Insertions at the C-terminal end of ERBB2 exon 20 induce a change in conformation of the α-C helix leading to a constitutively active formation which affects the drug-binding pocket [PMID: 29686424].
- **CBV Criteria:** The boundaries defined to capture ERBB2 exon 20 insertions as a class-based variant start at codon 770 and end at codon 783.

JAK2 exon 12 indel

- **Background:** Janus kinase 2 (JAK2) is a non-receptor membrane associated protein tyrosine kinase (PTK) made up of 1132 amino acids and located on chromosome 9p24. JAK2 belongs to the Janus kinase (JAK) family and has 25 exons—exons 3–7 encode the FERM domain, exons 9–11 encode the SH2-pseudokinase domain, exons 12–19 encode the kinase-like or pseudokinase domain (JH2), and exons 19–25 encode the C-terminal tyrosine kinase domain (JH1) [PMID: 23592968; PMID: 17639043; PMID: 29379470]. In addition, codons 523–544 in exon 12 encode the SH2-pseudokinase linker region and codons 817–839 in exon 19 encode the linker between the JH1 and JH2 protein kinase regions [PMID: 23592968, PMID: 17639043]. Mutations in JAK2 often target exon 12 including the region adjacent to the start of the pseudo-kinase domain [PMID: 21674578]. JAK2 exon 12 mutations (missense and in-frame deletions) are prevalent in myeloproliferative neoplasms including polycythemia vera [PMID: 21674578].
- **CBV Criteria:** The boundaries defined to capture JAK2 exon 12 in-frame insertions and deletions (indels) as a class-based variant start at codon 536 and end at codon 547. These boundaries capture the most common JAK2 exon 12 indels, including N542_E543del, E543_D544del, F537_K539delinsL and R541_E543delinsK mutations.

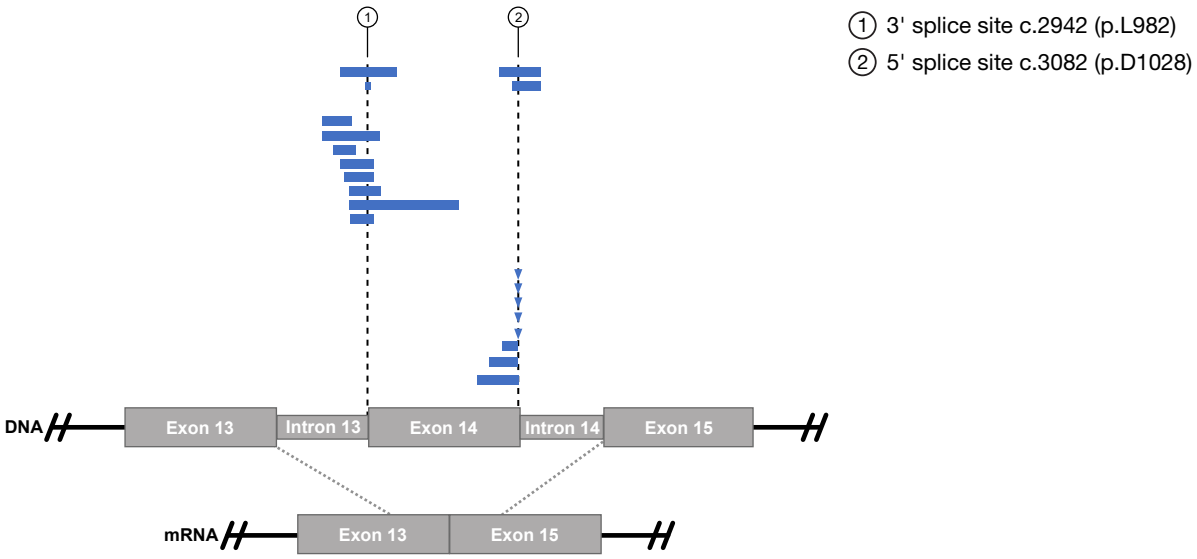
KIT exon 11 insertion and KIT exon 11 deletion

- **Background:** KIT, also known as CD117, is a proto-oncogene receptor tyrosine kinase made up of 976 amino acids located on chromosome 4q12 [PMID: 16689459]. KIT is a member of the PDGFR type III receptor tyrosine kinase family [PMID: 26579483; PMID: 26309392]. KIT has 21 exons—exons 1–9 encode the extracellular ligand-binding region containing five Ig-like domains, exon 10 encodes the transmembrane (TM) domain, and exon 11 encodes a cytoplasmic juxtamembrane region. The intracellular region of KIT is encoded by exons 12–21, wherein, exons 12–14 encode the tyrosine kinase domain 1, exons 15–16 encode the kinase insert (KI) region, and exons 17–19 encode the tyrosine kinase domain 2 [PMID: 24449920, PMID: 27536065, PMID: 31378162, PMID: 15342366, PMID: 17555444]. Recurrent somatic KIT alterations are observed in both solid and hematological cancers and include activating mutations such as single nucleotide variants (SNVs), small duplications, and complex in-frame insertions or deletions (indels). Mutations in KIT exons 8, 9, 11, and 17 disrupt the auto-inhibitory mechanisms and lead to constitutive activity [PMID: 27536065]. While mutations in exons 8 and 17 are common in myeloid cancers, 60–70% of somatic mutations (including SNVs and indels) in gastrointestinal stromal tumor (GIST) occur in exon 11, which encodes the juxtamembrane domain [PMID: 27536065, PMID: 22588877, PMID: 23678293; NCCN-GIST].
- **CBV Criteria:** The boundaries defined to capture KIT exon 11 insertions and deletions as a class-based variant start at codon 550 and end at codon 580.

MET exon 14 skipping

- **Background:** The MET proto-oncogene is a receptor tyrosine kinase made up of 1390 amino acids and is located on chromosome 7q31 [PMID: 15735036]. MET has 21 exons containing three main structural domains—an extracellular Sema domain in exon 2, a juxtamembrane domain in exon 14, and a tyrosine kinase domain in exons 15-21 [PMID: 28376232, PMID: 9380410]. Splice site mutations flanking exon 14 are observed in 4% of non-small cell lung cancer (NSCLC). These mutations include canonical splice site mutations affecting exon 14 and deletions that extend into the splicing motifs within intron 13 [PMID: 25971938, PMID: 27343443]. Such mutations disrupt splicing leading to the formation of an alternative transcript that joins exon 13 directly to exon 15 and skips exon 14 entirely. The MET exon 14 skipping transcript lacks the juxtamembrane domain that contains the recognition motif for ubiquitin-dependent proteolysis and thus leads to a marked increase in steady-state level of the MET protein [PMID: 28164087].

- **CBV Criteria:** The boundaries defined to capture MET exon 14 skipping as a class-based variant can be summarized into four individual rules: (a) has transcript = NM_001127500.3, exon = 14, and location = splicesite_3 (at c.2942) or splicesite_5 (at c.3082), (b) is an intronic deletion ≥ 4 bp impacting 30 nucleotides preceding exon 14 (c) is a missense variant at c.3082 [PMID: 27343443, PMID: 26729443, PMID: 25971938, PMID: 25898962, PMID: 25898965, PMID: 31472177], (d) is one of the following variants with confirmed skipping defined as c.3082delG [PMID: 25971938, PMID: 27343443], c.3066_3081delAGCTACTTTTCCAGAA, c.3075_3082del [PMID: 31472177], or c.3080_3081delAA [PMID: 26729443].



PDGFRA exon 18 deletion

- **Background:** Platelet derived growth factor receptor alpha (PDGFRA) is a cell surface tyrosine kinase receptor made up of 1089 amino acids and located on chromosome 4q12. PDGFRA contains 23 exons –exons 1-2 encode the signaling peptide, exons 3-10 encode the extracellular domain, exon 11 encodes the transmembrane domain, exon 12 encodes the juxtamembrane domain, and exons 13-23 encode the kinase domain [PMID: 33449152]. Mutations in PDGFRA often target the activation loop located within exon 18 and, to a lesser extent, target the juxtamembrane domain and the N-lobe of the kinase domain found within exons 12 and 14 [PMID: 30778083, PMID: 33449152, PMID: 15146165]. Activating PDGFRA exon 18 mutations such as V561D, N659K, D842V, and in-frame deletions are observed in 30-40% of KIT-negative gastrointestinal stromal tumors (GISTs) [PMID: 17193822, PMID: 15928335, PMID: 12522257, PMID: 23970477]. Specifically, PDGFRA in-frame deletions in codons 841-847 make up 23% of all exon 18 mutations [PMID: 15146165].
- **CBV Criteria:** The boundaries defined to capture PDGFRA exon 18 deletions as a class-based variant start at codon 841 and end at codon 847.

Copy-edit an analysis workflow to turn off CNV analysis

Use the following procedure to create a custom workflow with CNV analysis turned off. For more information, see the Ion Reporter™ Software help system or contact support.

1. Sign in to the Ion Reporter™ Software.
2. In the **Workflows** tab, select the checkbox in the row of the analysis workflow (page 58) to be copied, then click **(Actions)** ▸ **Copy**.
3. Click through each step in the workflow bar until you are in the **Copy Number** workflow step.
4. In the **Copy Number** step, select **No Baseline – Don't call CNVs** from the **Baseline** dropdown list.
5. Click **Next** to advance to the **Confirm** step, enter a **Workflow Name** and **Description** (*optional*), then click **Save Workflow**.

The customized analysis workflow is now available in the **Workflows** screen.

CNV analysis

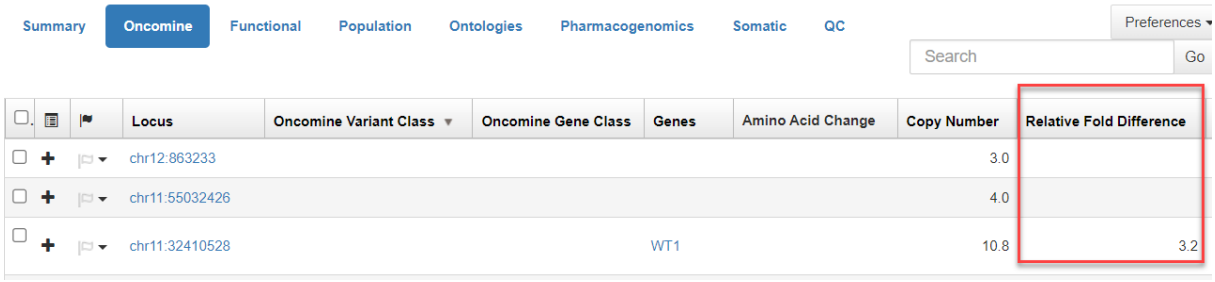
In Ion AmpliSeq™ assays, estimates of Copy Number Variation (CNV) are made by counting reads for each amplicon, making adjustments to account for variability, comparing those read counts to expected counts for the same amplicons in a "normal" sample, then making further adjustments.

To make a CNV call the following criteria must be met.

- MAPD <0.5
- CNV Ratio for a copy number gain must be above a threshold value. For more information, see the Ion Reporter™ Software help system or contact support.
- P-value $<10^{-5}$

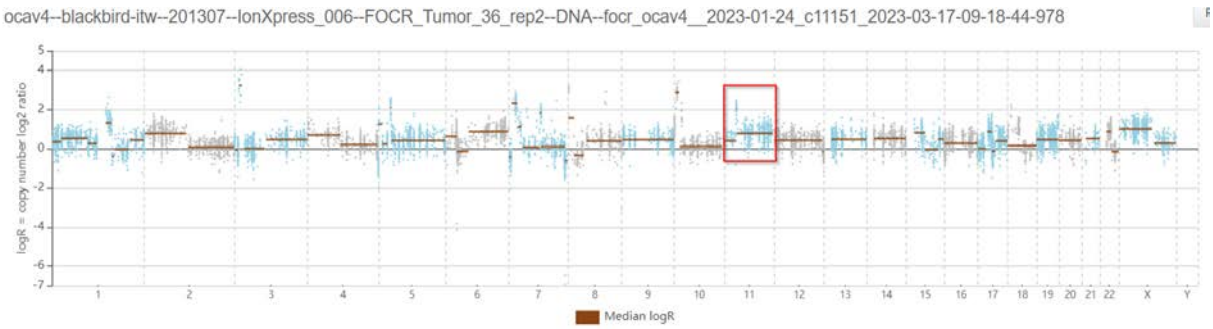
Relative Fold Difference

Oncomine™ Comprehensive Assay Plus workflows use a new parameter, relative fold difference (RFD), to adjust copy-number gain calls relative to the arm-level changes. RFD enables distinguishing between gene CNV calls due to arm-level and focal copy number changes.



The default value for RFD is 1.6. As a result, CNV gain calls with a RFD of 1.6 and higher are reported.

RFD is only applicable for CNV gain calls and reported only for cases of successful genomic segmentation assessment and determination of a chromosomal arm.



CNV somatic confidence range

The somatic CNV algorithms in Ion Reporter™ Software deliver not only a ploidy estimate call, but also a 90% confidence interval. The 5% lower confidence bound value is the ploidy estimate where there is 95% confidence that the true ploidy is above that value. The 95% upper confidence bound is the ploidy estimate where it is 95% certain that the true ploidy is below that value. For calling focal amplification, the lower bound is important and not the upper bound.

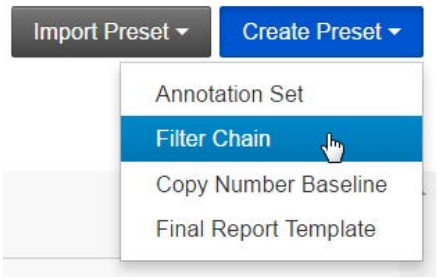
Note: The Oncomine™ Variant Annotator plugin annotates somatic CNVs in the Oncomine™ Comprehensive Assay Plus results. For more information, see the Ion Reporter™ Software help system.

How to change the confidence interval threshold default value

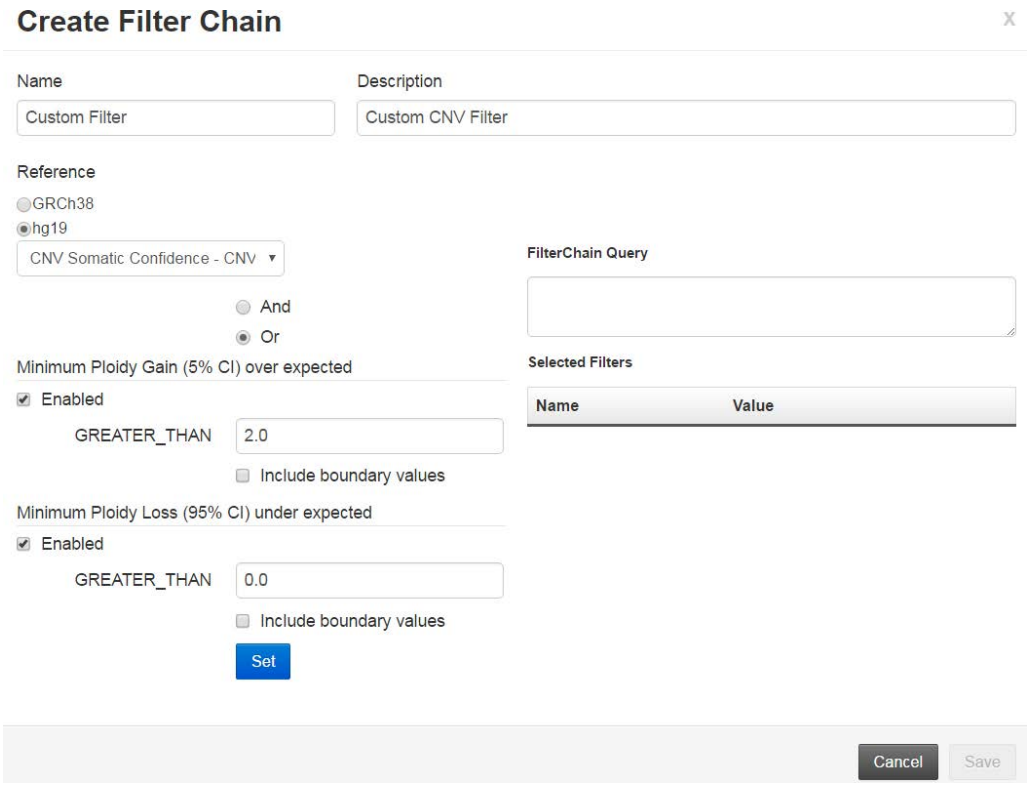
To change the confidence interval threshold default value, you must create a custom somatic CNV filter, then reanalyze the sample.

For more information about confidence interval threshold values, see the Ion Reporter™ Software help system or contact support.

1. Sign in to the Ion Reporter™ Software.
2. In the **Workflows** tab, in the **Presets** screen, click **Create Preset** ▸ **Filter Chain** .



3. Enter a **Name** *(required)*, and **Description** *(optional)*, for the new Filter Chain.



4. Ensure the **Reference** hg19 radio button is selected.

5. Select **CNV Somatic Confidence** - **CNVs Only** from the **Choose Filter** dropdown list.

6. Ensure **Enabled** is selected for both **Minimum Ploidy Gain (5% CI) over expected** and **Minimum Ploidy Loss (95% CI) under expected**.

Note: Deselect **Minimum Ploidy Loss (95% CI) under expected** to filter only for copy-gain genes.

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7. Enter your desired threshold values in the respective **GREATER_THAN** fields, then click **Set**.

Note: The new value is the ploidy of gain you want to threshold on when looking at the respective confidence interval value.

For example, setting the **Minimum Ploidy Gain (5% CI) over expected** threshold value to 1.0 causes the filter to look for all copy-gain genes whose 5% CI value is of ploidy 3 or greater.

8. Click **Save**.

Note: See the Ion Reporter™ Software help system for more information about creating complex filter chains.

In the image in step 3, the **Minimum Ploidy Gain (5% CI) over expected greater_than** is set to 2.0 (over expected normal), so copy-gain genes with ploidy >4 are filtered in. So if looking for copy-gain genes whose 5 CI value is anything over the expected normal of autosomes (2), leave the value set at 0.0. The **Minimum Ploidy Loss (95% CI) under expected to be greater_than** was set to 0.0 (under the expected normal), so only genes with a ploidy <2 are filtered in as copy-loss. For example, the following example CNV call confidence interval data result in the CNV data to be filtered in or out of the results:

- A gene with suspected gain with 5 CI = 4.1% and 95% CI = 10.3 is filtered in (5 CI >4).
- A gene with suspected loss with 5 CI = 0% and 95% CI = 1.0 is filtered in (95% CI <2).
- A gene with suspected gain with 5 CI = 2.2% and 95% CI = 3.6 is filtered out (5 CI 2.2<4% and 95% CI 3.6>2).
- A gene with 5 CI = 0.8% and 95% CI = 2.1 is filtered out (0.8 is less than 4 and 2.1 is greater than 2) (5 CI 0.8<4% and 95% CI 2.1>2).

CNV baseline creation

Known sources of variability of CNV estimation include pool imbalance (when the assay has more than one pool of amplicons), total number of reads and per amplicon attributes of GC proportion, and length of the amplicon insert. In practice, we observe other variability that does not associate with known attributes yet is systematic. The method that we use trains on many diverse samples, captures systematic effects, and encodes these into a file (the "baseline").

When augmenting a baseline, new samples are run, the size of each systematic effect encoded in the baseline is estimated, and a correction is applied to remove the effect. These added samples need not be normal, and should be diverse to capture likely systematic variation.

The following instructions walk you through using the new Variability Correction Information Baseline (VCIB) CNV baseline, creating a new VCIB CNV baseline, or augmenting an existing VCIB CNV baseline for Oncomine™ Comprehensive Assay panels.

Use VCIB CNV baseline

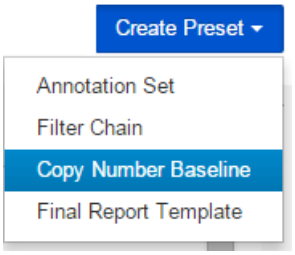
If you want to use the VCIB CNV baseline included in Ion Reporter™ Software 5.20, simply select it when creating your workflow.

Note: The VCIB CNV baseline is currently noncompatible with the Ion GRCh38 human reference.

Create a CNV baseline

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

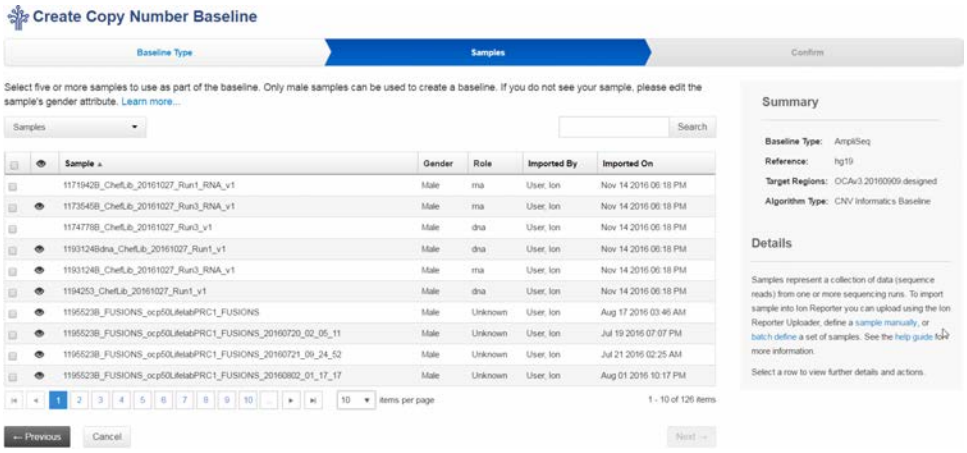
1. In the **Workflows** tab, in the **Presets** screen, click **Create Preset**, then select **Copy Number Baseline** from the dropdown list.



2. Click **AmpliSeq**, select **Oncomine™ Comprehensive Plus DNA Regions v1.5** as your Target Regions file, then click **Next**.

3. Select at least 48 samples, flag at least 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 panel to see your totals.



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

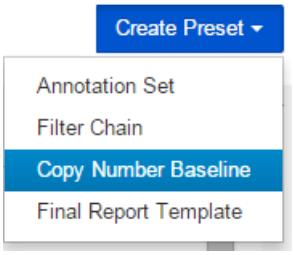
Note: Log files for both successful and failed analyses include the **BaselineCreation.log** file, which has the BAM files named that were rejected due to similarity to other files in the baseline, as well as the **map.TmapMergeActor-00.err** file that has the BAM files named that were rejected due to QC failure.

To add this new baseline to your workflow, proceed to “Create an Ion Reporter™ Software analysis workflow” on page 119.

Augment (add Samples to) an existing VCIB CNV baseline

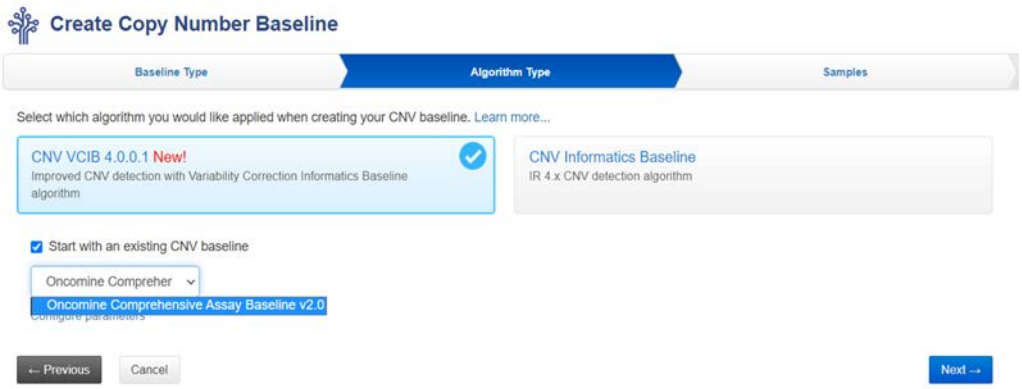
Ion Reporter™ Software provides a wizard to guide you through Copy Number Variation (CNV) baseline creation. This example describes how to add additional samples to an existing CNV baseline.

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



2. Click **AmpliSeq**, select **Oncomine™ Comprehensive Plus DNA Regions v1.5** as your **Targets Region** file, then click **Next**.

3. Select the **Start with an existing CNV Baseline** checkbox, then select a baseline from the dropdown list.

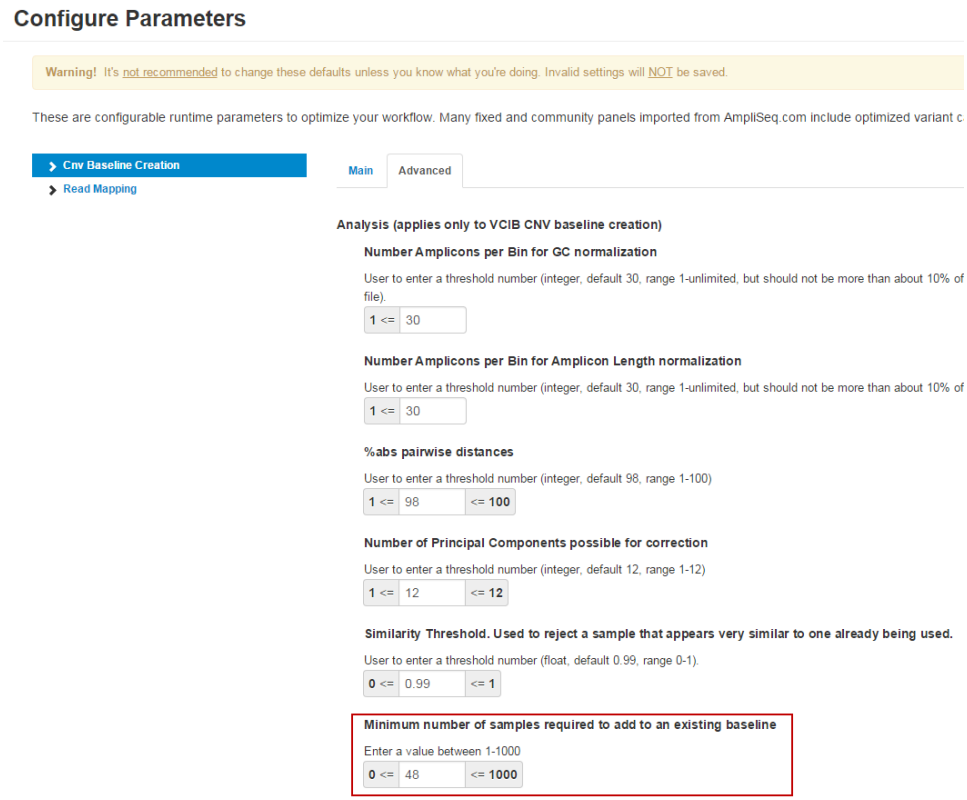


Note: By default, the software prompts you to add another 48 samples. However, you can set the number to 1 or more. Add non-Normal samples. Marking samples as "Normal" in the augmentation workflow has no effect, only the original Normals in the first baseline creation are treated as Normals in the augmented baseline.

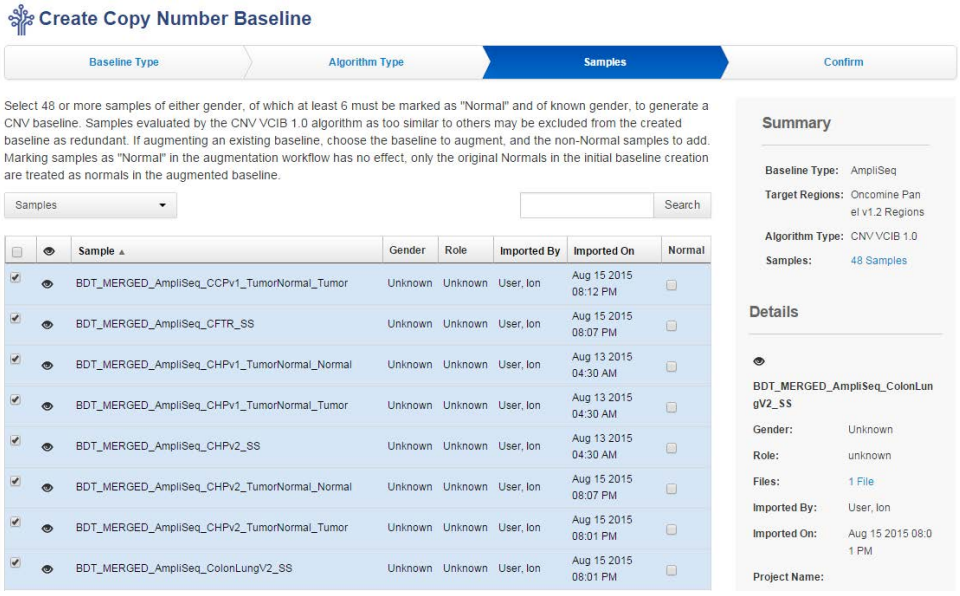
---

4. Click the **Configure Parameters** link.

5. In the **Configure Parameters** dialog box, click **Cnv Baseline Creation**, then **Advanced**. Set the **Minimum number of samples required to add to an existing baseline** to the number you are adding, click **Done**, then click **Next**.



6. Select additional samples, then click **Next**.

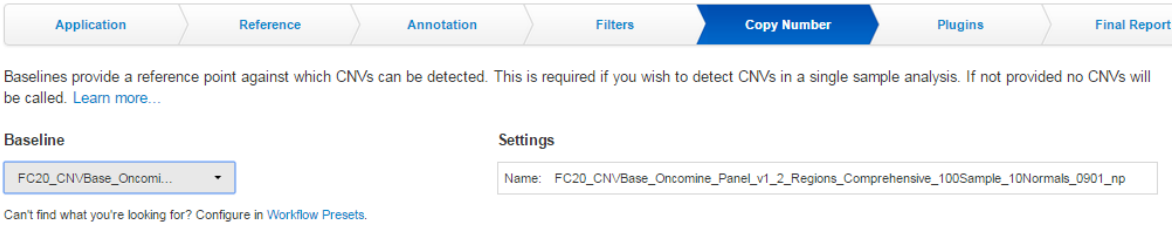


7. Enter a name for your baseline, then click **Create Baseline** to save.

Create an Ion Reporter™ Software analysis workflow

Ion Reporter™ Software provides a wizard to guide you through creating a workflow. However, it can be easier to copy an existing Oncomine™ workflow and edit it by adding your newly created baseline.

1. In the **Workflows** tab, in the **Overview** screen, select an appropriate Oncomine™ Comprehensive workflow.
2. In the **Details** pane, click **Actions** ▸ **Copy**.
   The workflow wizard opens to the **Research Application** step in the **Create** screen.
3. Click **Next** to advance to the **Reference** step.
4. Confirm that **hg19** is the selected **Reference**, then select a **Target Regions**, **Hotspot Regions**, and **Fusions** BED file from the respective dropdown lists. Click **Next**.
5. Select an **Annotation Set** from the dropdown list, then click **Next**.
6. Select a **Filter Chain** from the dropdown list, then click **Next**.
7. In the **Copy Number** step, select the baseline that you want to use from the **Baseline** dropdown list, then click **Next**.



8. In the **Plugins** step, ensure that all **In-Analysis** plugins are deselected, then click **Next**.
9. Select a **Final Report Template** from the dropdown list, then click **Next**.
10. In the **Parameters** step, review the default settings, then click **Next**.

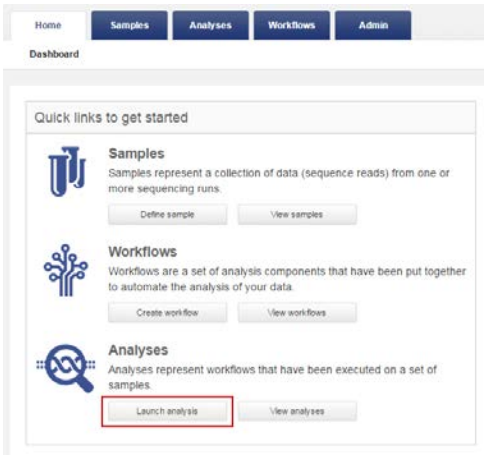
Note: Although **Read Mapping** parameters are exposed in workflow creation, it is not necessary to change any settings.

---

11. In the **Confirm** step, enter a **Workflow Name** and **Description**, then click **Save Workflow**.

Launch an analysis

1. In the **Home** tab, in the **Dashboard** screen, click **Launch Analysis**.

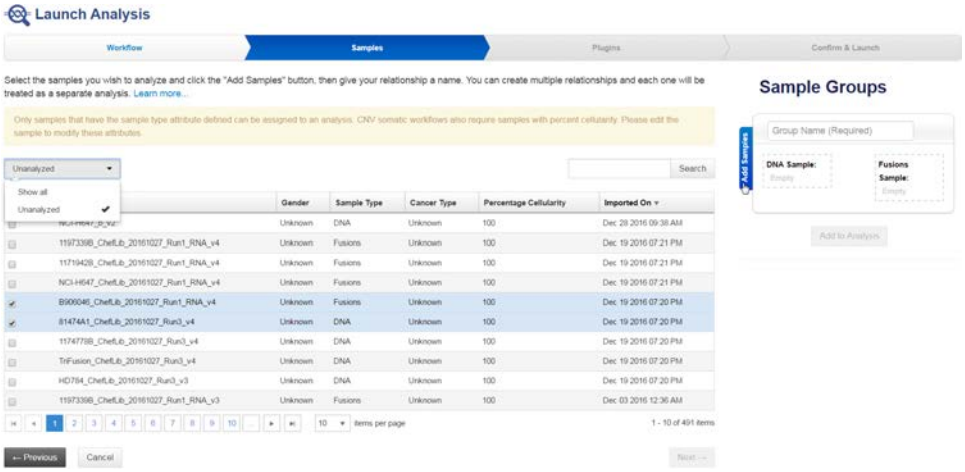


2. In the **Launch Analysis** wizard, in the **Workflow** step, select your custom workflow or one of the pre-installed Oncomine™ workflows, then click **Next**.

3. Select the samples to include in the analysis.
   a. Use the **Samples** dropdown list to filter the available samples.

   b. Click within a sample row to select each sample to include in a sample group, then click **Add Samples** in the **Sample Groups** pane.

   c. Enter a **Group Name**, then click **Add to Analysis**.

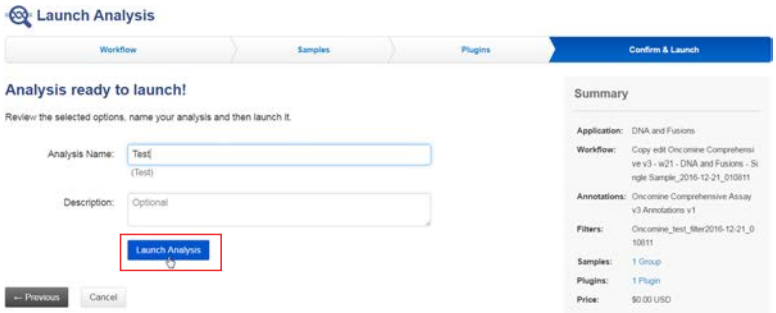


Note: The **Percentage Cellularity** sample attribute is required for DNA samples.

4. Repeat substep 3b and substep 3c to add additional **Sample Groups**.

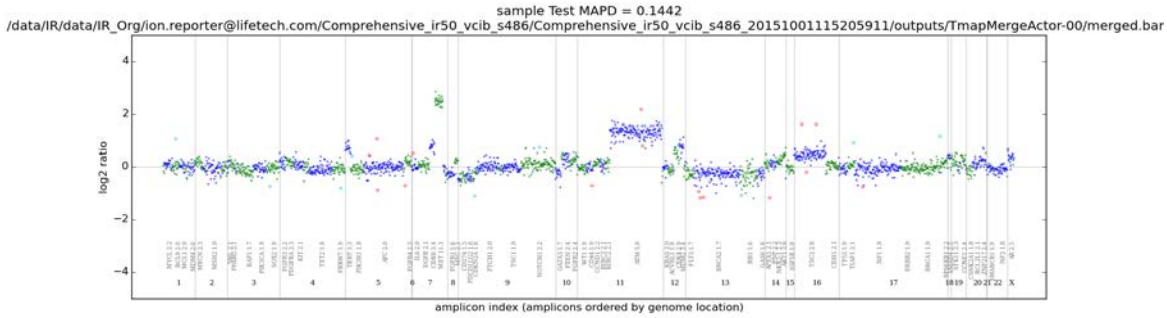
5. Click **Next** 2 times to move to the **Confirm & Launch** step.

6. In the **Confirm & Launch** step, enter an **Analysis Name** and **Description** for the analysis, then click **Launch Analysis**.



7. Review your results in the **Analyses** tab. Confirm the CNV workflow and baseline used in the **Details** pane.

8. *(Optional)* Download the analysis results, then review the results visually with the cn_results.png.



Interpretation example: This plot shows log2 ratios across the genome and highlights panel CNV IDs. The alternating blue and green color is used to distinguish between adjacent CNV IDs. The outliers data are the small pink circles. The numbers on the X axis are the chromosomes. Above this are the CNV ID names and the mean CN call for each CNV ID. You can see copy number gains on chromosome 7 and chromosome 11. The MAPD number at the top of the plot is a QC metric measuring the noisiness of the sample. A low MAPD is good. MAPD >0.5 is considered to fail QC. Below the MAPD is the BAM file name. Log2 ratios of 0 are equivalent to a copy number call of 2 (normal). If the sample was a male, you would expect to see a copy number of 1 on chromosome X.

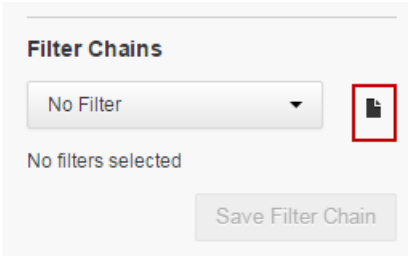
D Subset filter creation

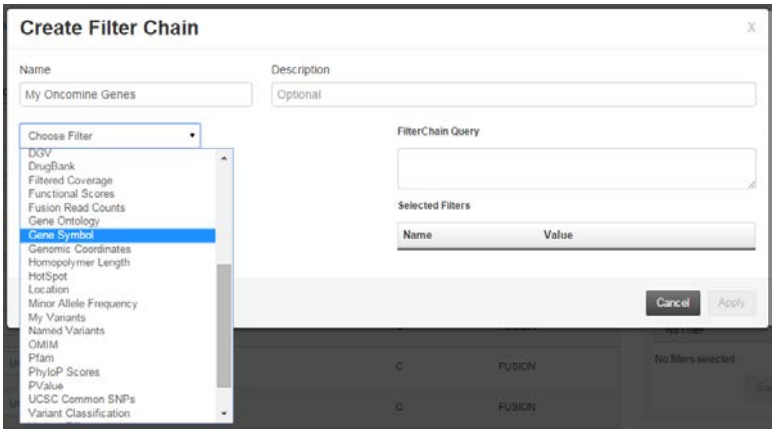
If you do not want to generate information on all the Oncomine™ variants, you can create subset filters to look at only certain genes and variants. This appendix provides examples for creating subset filters.

Create a gene-level filter

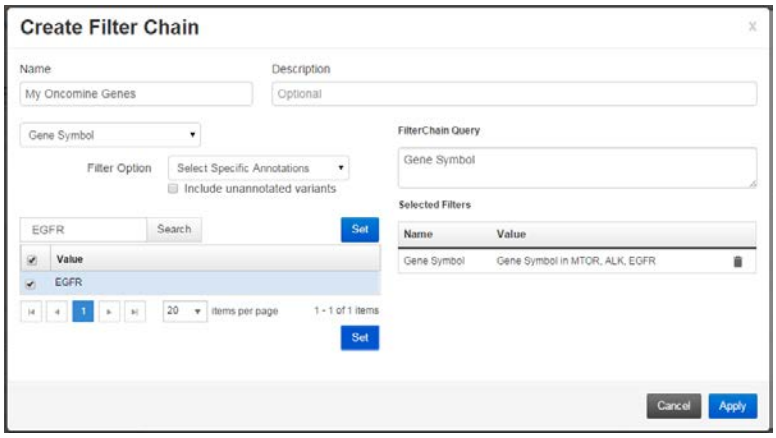
If you just want to filter for a set of genes in your Oncomine™ analyses, applying the Gene Symbol filter is the easiest approach.

1. In the **Analyses** tab, in the **Overview** screen, click on the desired **Analysis** hyperlink in the **Analyses** table.
2. In the **Filter Options** pane, click **(New)**.
3. In the **Create Filter Chain** dialog box, enter a **Name** and **Description** for your gene-level filter.
4. Click **Choose Filter**, then select **Gene Symbol** from the dropdown list.





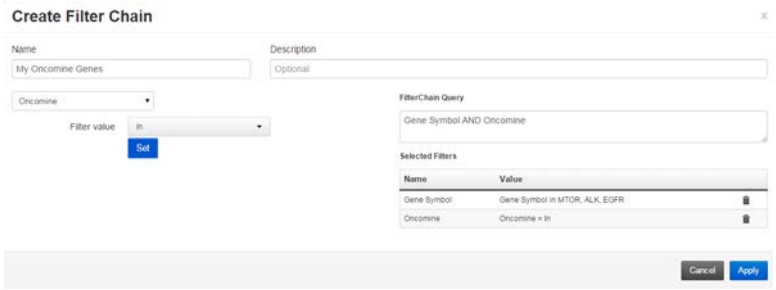
5. In the **Search** field, enter a gene symbol of interest (for example, EGFR), then click **Go** or press Enter.



6. Select each search result to be included, then click **Set**. Repeat step 5 and step 6 to add additional **Gene Symbols**.

7. In the **Choose Filter** dropdown list, select **Oncomine™**.

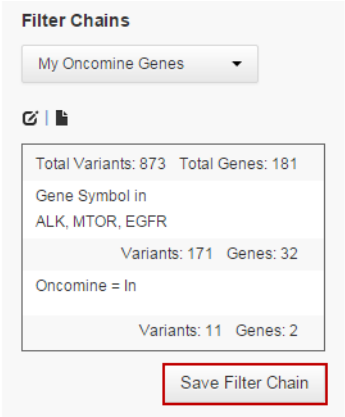
8. In the **Filter value** dropdown list, select **In**, then click **Set**.



9. Click **Apply**.

10. In the **Filter Options** pane, click **Save Filter Chain**.

    Your new filter is now available for use.



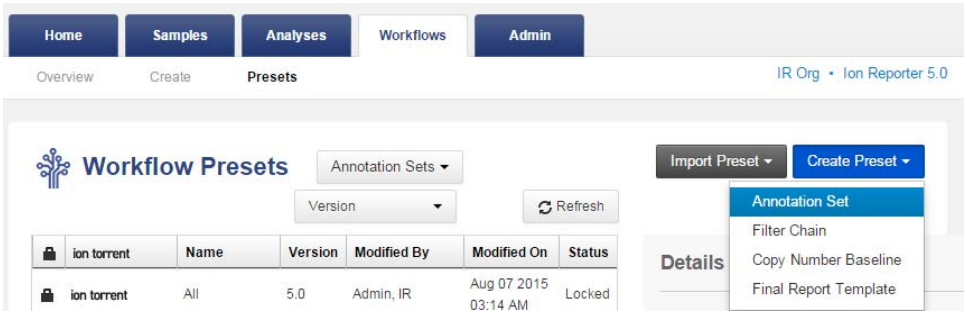
Next, copy a relevant workflow, then select this new filter chain as the default.

Create a variant-level filter

If you only want to review a subset of variants from the <assay name>_variantDB.vcf file, you must first create a new workflow in the Ion Reporter™ Software. To create a new workflow you must first prepare a custom variantDB file and new annotation set, then copy and edit an existing workflow to use the newly created custom variantDB file and annotation set.

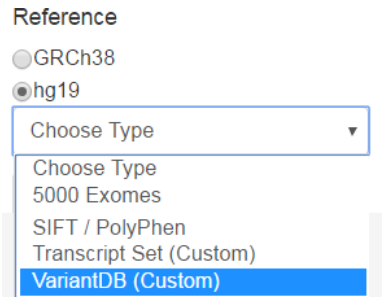
Create a new variantDB from the provided file

1. In the **Workflows** tab, in the **Presets** screen, click **Create Preset** ▸ **Annotation Set**.



2. In the **Create Annotation Set** dialog box, enter a **Name**, and **Description** for your **Annotation Set**.

3. Click **Choose Type**, then select **VariantDB (Custom)** from the dropdown list.

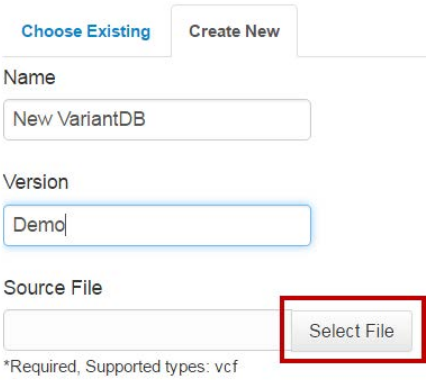


4. In the **Create New** tab, enter a **Name**, and **Version** for the new variantDB.

5. Click **Select File**.

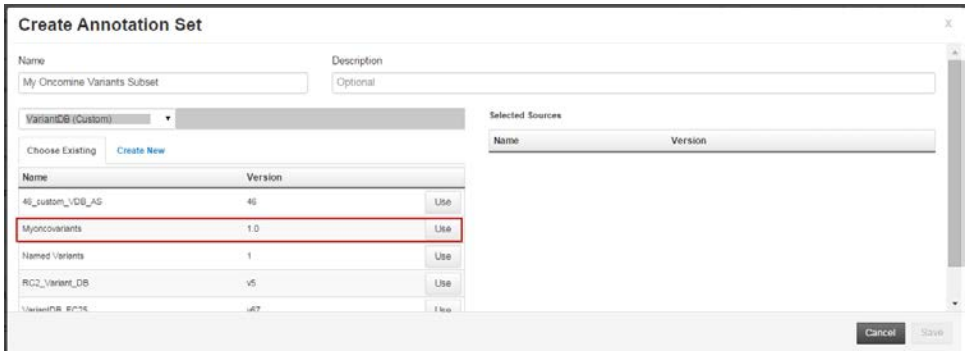
6. Navigate to the <assay name>_variantDB.vcf file, click **Open**, then click **Upload**.

7. Click **Save**.
   Your new variantDB is now available in the **Workflow Presets** table in the **Presets** screen.



Create a new annotation set from the new variantDB and existing Oncomine™ annotation sources

1. In the **Workflows**tab, in the **Presets** screen, click **Create Preset ▸ Annotation Set**.
2. In the **Create Annotation Set** dialog box, enter a **Name** and **Description** for your new **Annotation Set**.
3. Click **Choose Type**, then select **VariantDB (Custom)** from the dropdown list.
4. In the **Choose Existing** tab, scroll down, select the variantDB file that you previously created, then click **Use**.



5. In the **Choose Type** dropdown list, select **5000Exomes**, then click **Use**. Repeat for the following sources:
   - ClinVar
   - dbSNP
   - DGV
   - DrugBank
   - Gene Ontology
   - Pfam
   - PhyloP Scores
   - RefGene Functional Canonical Transcripts Scores
   - RefSeq GeneModel
   - Oncomine™ Canonical Transcripts

   Click **Use** after selecting each.

Note: Some annotation sets are under existing selections in the dropdown list:

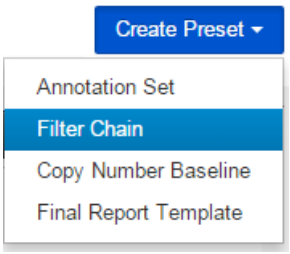
- **RefGene Functional Canonical Transcripts Scores** is under **SIFT/PolyPhen**
- **RefSeq GeneModel** is under **Gene Model**
- **Oncomine™ Canonical Transcripts** is under **Transcript Set (Custom)**

6. Click **Save**.

Your new **Annotation Set** is now available in the **Workflow Presets** table in the **Presets** screen.

Create a new filter chain using the new variantDB

1. In the **Workflows** tab, in the **Presets** screen, click **Create Preset ▸ Filter Chain**.

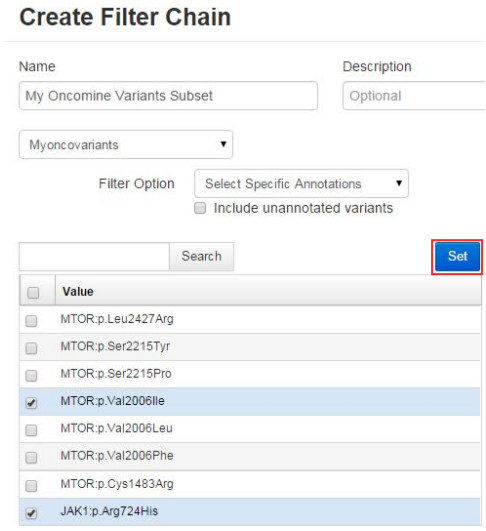


2. In the **Create Filter Chain** dialog box, enter a **Name** and **Description** for your new **Filter Chain**.

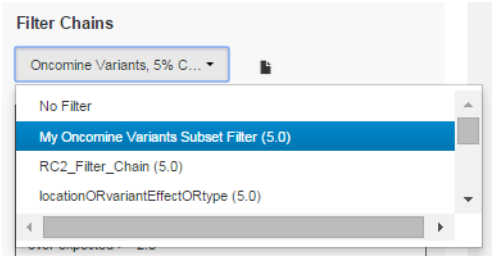
3. Click **Choose Filter**, then select the variantDB you created in the dropdown list.

4. Select the variants that you want to filter in, then click **Set**.

5. Click **Save**.



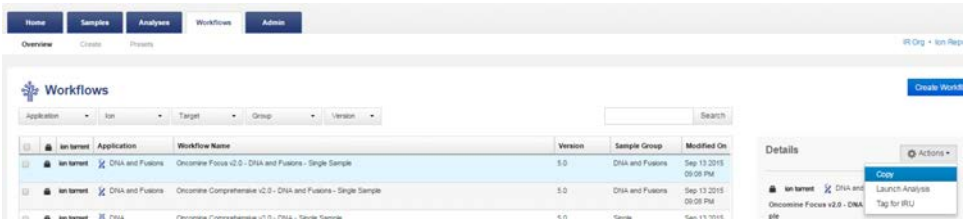
You can now apply your new variant subset filter to analyses.



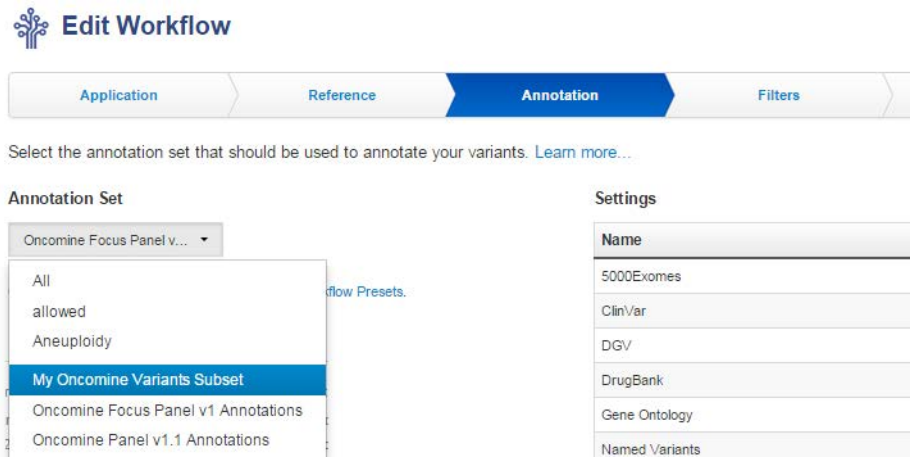
Create a copied workflow with the new annotation set and filter chain

Now you need to create a new workflow to use the annotation set and filter chain that you created.

1. In the **Workflows** tab, in the **Overview** screen, select the workflow to be copied, then click **Actions** ▸ **Copy** in the **Details** pane.



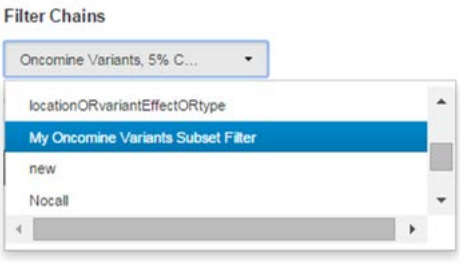
2. In the **Annotation** step, add the new annotation set.



3. In the **Filters** step, add the new filter chain.

4. In the Confirm step, name the workflow.

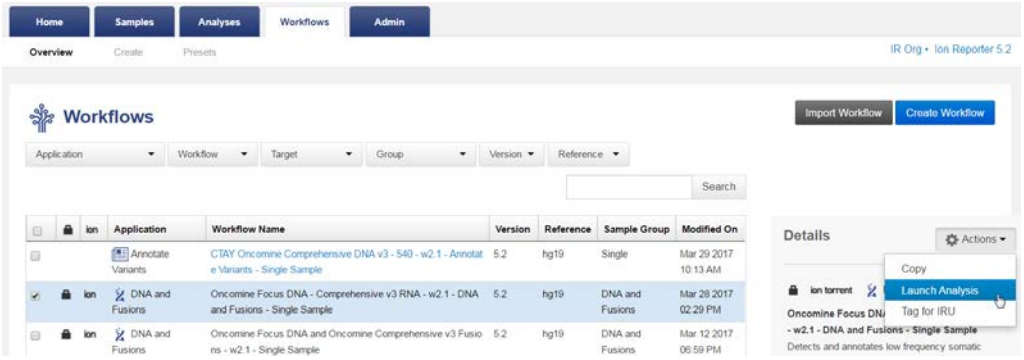
5. Click **Save Workflow**.
   Your new workflow is now available for use in the **Workflows** tab.



Use the new workflow

Your variants subset workflow is now ready for use.

Select your new workflow, then click **Actions** ▸ **Launch Analysis**.



Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Chemical safety

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

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

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)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

Documentation and Support

Related documentation

Document	Description
Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide (Pub. No. MAN0013432)	Describes the automated preparation and templating of Oncomine™ Comprehensive Assay Plus libraries using the Ion Chef™ System.
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 550™ Kit – Chef User Guide (Pub. No. MAN0017275)	Describes the automated template preparation of Oncomine™ Comprehensive Assay Plus libraries using the Ion Chef™ System for sequencing on the Ion S5™ System.
Ion Library TaqMan™ Quantitation Kit User Guide (Pub. No. MAN0015802)	Provides detailed instruction and troubleshooting for use of the Ion Library TaqMan™ Quantitation Kit.
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.
Ion Reporter™ Software 5.20 User Guide MAN0028321	Comprehensive instruction for use of Ion Reporter™ Software.

Note: For additional documentation, see “Customer and technical support” on page 132.

Customer and technical support

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

- Worldwide contact telephone numbers
- Product support information
  - Product FAQs
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- 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**.