ion torrent

Oncomine™ Tumor Mutation Load Assay

USER GUIDE

Catalog Numbers A37909, A37910

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Revision D.0

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

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Revision history: MAN0017042 D.0 (English)

Revision	Date	Description
D.0	26 January 2024	• Update Ion AmpliSeq™ Chef Supplies DL8 (Part No. A29027) to include the PCR Plate Frame.
C.0	4 October 2018	• Include troubleshooting information for FFPE DNA samples with high levels of deamination • Include Uracil DNA Glycosylase (UDG) treatment supplemental protocol
B.0	31 August 2018	• Updated Mutation Load calculation • Updated to include information for use with the Ion 550™ Chip • Updated for use with Ion Reporter™ Software 5.10
A.0	20 December 2017	Oncomine™ Tumor Mutation Load Assay User Guide, provides instruction for library preparation, templating, sequencing, and results analysis of the Oncomine™ Tumor Mutation Load Assay.

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

Contents

1 Product information

Definition of Mutation Load

Mutation Load, also known as Tumor Mutational Burden (TMB), as determined by the Oncomine™ Tumor Mutation Load Assay is defined as the number of nonsynonymous variants (missense and nonsense single nucleotide variants (SNVs)), plus insertion and deletion variants (INDELs) detected per megabase (Mb) of exonic sequence. The Ion Reporter™ Software TMB algorithm filters out germline mutations using the Mutation Load Calculation Filter Chain when calculating the Mutation Load result.

Table 1 Mutation Load algorithm versions and descriptions

Version[1]	Description
TMB algorithm v2.5	Used in the Oncomine™ Tumor Mutation Load - w2.0 - DNA - Single Sample workflow. The Mutation Load Calculation Filter Chain parameter = Mutation Load (Somatic Mutations) by default. precalibration Mutation Load = $\left(\frac{\text{Nonsynonymous Somatic Mutations} \times 10^6}{\text{Total Exonic Bases with Sufficient Coverage}}\right)$ IF precalibration Mutation Load ≥25, THEN Mutation Load = (precalibration Mutation Load – 25) x Calibration Slope + 25 IF precalibration Mutation Load <25, THEN no calibration is required Mutation Load = precalibration Mutation Load x 1.0 • Includes exonic INDELs in the Mutation Load calculation. • Calibration factor applied to the Mutation Load calculation. **Note:** To enable other workflows to apply the TMB algorithm v2.5 you must copy then edit the Oncomine™ Tumor Mutation Load - w2.0 - DNA - Single Sample workflow to match the workflow of choice (for example, Oncomine™Comprehensive v3 - w3.2 - DNA and Fusions - Single Sample). For more information, see “Enable tumor mutational burden calculation in existing analysis workflows” on page 49.

Table 1 Mutation Load algorithm versions and descriptions *(continued)*

Version[1]	Description
TMB algorithm v2.0	Used in workflows enabled by the user to include the mutation load calculation by selecting a Mutation Load Calculation Filter Chain parameter[2]. Mutation Load = (Nonsynonymous Somatic Mutations x 10^6) / Total Exonic Bases with Sufficient Coverage • Includes only exonic nonsynonymous somatic SNVs in the Mutation Load calculation. INDELs are not included in the Mutation Load calculation even if the selected filter chain filtered-in INDELs. • The Mutation Load Calculation Filter Chain parameter can be applied to any "DNA - Single Sample" or "DNA and Fusions - Single Sample" workflow.
TMB algorithm v1.0[3]	Used in the **Oncomine™ Tumor Mutation Load - w1.0 - DNA - Single Sample** workflow. Includes all exonic and intronic synonymous and nonsynonymous somatic SNVs in the Mutation Load calculation.

[1] To determine the TMB algorithm version used for analysis of a particular sample, look in the `statistic.txt` file. For more information, see page 34.

[2] We recommend setting the **Mutation Load Calculation Filter Chain** parameter to "Mutation Load (Somatic SNVs)" filter chain. For more information, see the Ion Reporter™ Software online help.

[3] Hidden from view in Ion Reporter™ Software 5.6.

Note: Reanalysis of samples previously run with TMB algorithm v1.0 (or v2.0) with the new Ion Reporter™ Software 5.10 TMB algorithm v2.5 may result in a different Mutation Load result due to changes in the algorithm.

Product description

The Oncomine™ Tumor Mutation Load Assay is a targeted next-generation sequencing (NGS) assay that is designed to accurately evaluate tumor mutational burden (mutations/Mb) and generate tumor profiles by annotating cancer driver variants in a simple sample-to-answer workflow. The assay detects and annotates low frequency somatic variants (SNPs and INDELs) from 409 genes, spanning ~1.7 Mb of genomic space, encompassing 1.2 Mb of exonic sequence. This assay is designed to facilitate successful selection and identification of samples most likely to derive responses in cancer immunotherapy research.

This guide covers library preparation from formalin-fixed paraffin-embedded (FFPE) tumor samples—10 ng of DNA per primer pool—using the Oncomine™ Tumor Mutation Load Assay library preparation kits (manual and Chef-Ready), through results analysis. The assay is used with barcoded adapters so that multiple libraries can be combined and loaded onto a single chip to minimize the per-sample sequencing cost.

Barcoded libraries per chip	
Ion 540™ Chip	**Ion 550™ Chip[1]**
2–8[2]	2–16[2]

[1] The Ion 550™ Chip is only compatible with the Ion GeneStudio™ S5 Plus Sequencer, Ion GeneStudio™ S5 Prime Sequencer, or Ion S5™ XL Sequencer. The Ion 550™ Chip cannot be sequenced on the Ion S5™ Sequencer, or base model Ion GeneStudio™ S5 Sequencer.

[2] Calculation of the Mutation Load alone does not require as many reads as variant profiling. We recommend multiplex sequencing of no more than 4 libraries (Ion 540™ Chip)—6 libraries (Ion 550™ Chip)—to achieve sufficient read depth for variant calls at a ≥5% allele frequency.

The Ion Reporter™ Software 5.12 analysis workflow uses a custom variant calling and germline variant filtering algorithm to accurately estimate somatic variants in cancer research samples, with no matched normal sample required. The software provides a detailed report, feature-rich visualization, annotation of low frequency cancer driver variants (SNVs and INDELs), and the normalized Mutation Load (mutations/Mb). Included in the results are the percentage of mutations consistent with UV damage, tobacco smoke damage, deamination, and specific substitutions.

This guide covers the following products:

- Oncomine™ Tumor Mutation Load Assay – Manual primer panel (2-pools) (Cat. No. A37907)
- Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990)
- Ion Xpress™ Barcode Adapters (various Cat. Nos.)
- IonCode™ Barcode Adapters (Cat. No. A29751)

Note: Oncomine™ Tumor Mutation Load Assay – Chef-Ready Library Preparation (Cat. No. A37910) is also available for automated library preparation (see page 10). The kit provides the Oncomine™ Tumor Mutation Load Assay – Chef-Ready primer panel (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™ Tumor Mutation Load Assay – Manual Library Preparation Kit

The Oncomine™ Tumor Mutation Load Assay – Manual Library Preparation Kit (Cat. No. A37909) consists of the Oncomine™ Tumor Mutation Load Assay – Manual Library Preparation primer panel (2-pools), and the Ion AmpliSeq™ Library Kit Plus. Sufficient reagents are provided for the rapid preparation of 24 barcoded sample libraries from DNA.

Contents	Amount	Storage
Oncomine™ Tumor Mutation Load Assay – Manual Library Preparation primer panel (Part No. A37907, 24 reactions)		
Oncomine™ Tumor Mutation Load Assay (2X) (blue cap) (pool 1 of 2)	120 µL	–30°C to –10°C
Oncomine™ Tumor Mutation Load Assay (2X) (blue cap) (pool 2 of 2)	120 µL	
Ion AmpliSeq™ Library Kit Plus (Part No. 4488990)		
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™ Tumor Mutation Load Assay – Chef-Ready Library Preparation

The Oncomine™ Tumor Mutation Load Assay – Chef-Ready Library Preparation Kit (Cat. No. A37910) provides the Oncomine™ Tumor Mutation Load Assay – Chef-Ready Library Preparation primer panel at 2X concentration premeasured 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 sufficient for preparing 32 libraries. See the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432) for information about preparing Oncomine™ Tumor Mutation Load Assay libraries on the Ion Chef™ System.

Component	Amount	Storage
Oncomine™ Tumor Mutation Load Assay – Chef-Ready (Part No. A37908, 32 reactions)		
Oncomine™ Tumor Mutation Load Assay – Manual (2X) (pool 1 of 2)	4 × 150 µL	–30°C to –10°C
Oncomine™ Tumor Mutation Load Assay – Manual (2X) (pool 2 of 2)	4 × 150 µL	
Ion AmpliSeq™ Kit for Chef DL8 (Cat. No. A29024)		
Ion AmpliSeq™ Chef Reagents DL8 (Part No. A29025)	4 cartridges	–30°C to –10°C
Ion AmpliSeq™ Chef Solutions DL8 (Part No. A29026)	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	1 box 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)	1 set of 4 plates	15°C to 30°C

[1] Ion AmpliSeq™ Chef Solutions DL8 cartridges are shipped at ambient temperature, but must be stored at 2°C to 8°C on arrival.

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
IonCode™ Barcode Adapters 1–384 Kit OR Ion Xpress™ Barcode Adapters	A29751 4471250, 4474009, 4474518, 4474519, 4474520, 4474521, or 4474517
Agencourt™ AMPure™ PCR Purification kit	Beckman Coulter, A63880 or A63881
One of the following: • GeneAmp™ PCR System 9700 or Dual 96-well Thermal Cycler • AB™ 2720 Thermal Cycler • Veriti™ 96-Well Thermal Cycler • ProFlex™ 96-well PCR System	See web product pages
MicroAmp™ Optical 96-Well Reaction Plate	N8010560 4306737 (with barcode)
MicroAmp™ Fast Optical 96-Well Reaction Plate	4346907
MicroAmp™ Optical Adhesive Film	4311971
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 (not DEPC-Treated)	AM9932
Absolute ethanol	**MLS**
Pipettors, 2–200 µL, and low-retention filtered pipette tips	**MLS**

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	
Real-time PCR instrument (e.g.,Applied Biosystems™ 7900HT, 7500, StepOne™, StepOnePlus™, ViiA™ 7 Systems, or QuantStudio™ 12K Flex Real–Time PCR System)	See web product pages
96-well plate centrifuge	MLS
Qubit™ 4 Fluorometer[1]	Q33238
Recommended for nucleic acid isolation	
RecoverAll™ Multi-Sample RNA/DNA Workflow	A26069
RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	AM1975
MagMAX™ FFPE Total Nucleic Acid Isolation Kit	4463365
Recommended for nucleic acid quantification	
Qubit™ dsDNA HS Assay Kit (DNA)	/
Recommended for library quantification	
Ion Library TaqMan™ Quantitation Kit	4468802
Recommended for Uracil-DNA Glycosylase treatment	
Uracil-DNA Glycosylase (UDG), heat labile	78310100UN

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

Ion GeneStudio™ S5 Series instrument reference

In this document, Ion GeneStudio™ S5 Series Sequencer or Ion GeneStudio™ S5 Series System refers generically to the following systems, unless otherwise specified.

- Ion GeneStudio™ S5 System (Cat. No. A38194)
- Ion GeneStudio™ S5 Plus System (Cat. No. A38195)
- Ion GeneStudio™ S5 Prime System (Cat. No. A38196)

2 Before you begin

Procedural guidelines

- Minimize freeze-thaw cycles of panels by aliquoting as needed for your experiments. Panels can be stored at 4°C for 1 year.
- Use good laboratory practices to minimize cross-contamination of products. If possible, perform PCR setup in an area or room that is free of amplicon contamination. Always change pipette tips between samples.
- Use a calibrated thermal cycler specified in “Required materials not supplied”.
- 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™–96 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.

3 Library preparation

IMPORTANT! Oncomine™ Tumor Mutation Load Assay – Chef-Ready Library Preparation Kit (Cat. No. A37910) users.

- For instructions to prepare Oncomine™ Tumor Mutation Load Assay libraries on the Ion Chef™ System, see the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432).
- When templating the combined libraries, use at 50 pM concentration.

Ion Chef™ Instrument setup information for Chef Ready kit users

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

Stating material	# of primer pools	Target amplification cycles	Anneal & extension time
FFPE DNA	2	16	16 minutes

Set up DNA target amplification reactions

Guidelines for DNA isolation and quantification

- We recommend the RecoverAll™ Multi-Sample RNA/DNA Workflow (Cat. No. A26069) for isolating gDNA.
- We recommend the TaqMan™ RNase P Detection Reagents Kit (Cat. No. 4316831) for quantifying amplifiable human genomic DNA (see *Demonstrated Protocol: Sample Quantification for Ion AmpliSeq™ Library Preparation Using the TaqMan™ RNase P Detection Reagents Kit* (Pub. No. MAN0007732) available at **thermofisher.com**).
- The Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) can also be used for quantification, particularly for FFPE DNA, and highly degraded DNA samples.
- Quantification methods such as densitometry (for example, using a NanoDrop™ spectrophotometer) are not recommended, because they are not specific for DNA. Use of these methods can lead to gross overestimation of the concentration of sample DNA, under-seeding of the target amplification reaction, low library yields, and poor chip loading.

Guidelines for the amount of DNA needed per target amplification reaction

- For each target amplification reaction, use 300–30,000 copies of DNA (10 ng of mammalian gDNA) from normal or FFPE tissue.
- Increasing the amount of DNA results in higher-quality libraries, especially when DNA quality or quantity is unknown. We recommend that you use 1 ng gDNA (300 copies) only with high-quality, well-quantified samples.

Prepare DNA target amplification reactions

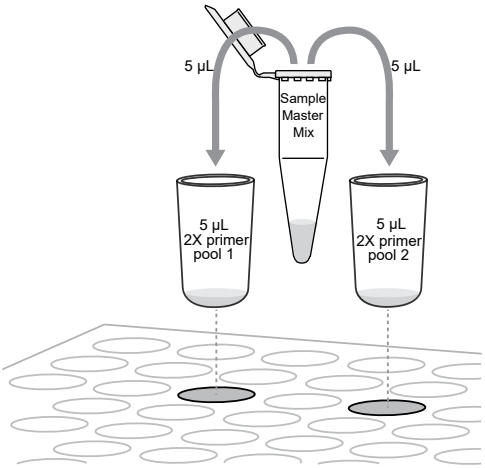
IMPORTANT! Primer pools and 5X Ion AmpliSeq™ HiFi Mix are viscous. Pipet slowly and mix thoroughly.

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)[1]	≤7.5 µL
Nuclease-free Water	to 12.5 µL

[1] Substitute 5 µL AcroMetrix™ Oncology Hotspot Control to prepare a control library.

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.
4. Add 5 µL of Oncomine™ Tumor Mutation Load Assay (2X) manual library preparation primer pool 1 into the first well, and 5 µL of primer pool 2 to the second well.
5. Seal the plate with a MicroAmp™ 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.



Amplify the 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™ 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
15 Cycles	Denature	99°C	15 sec
	Anneal and extend	60°C	16 min
Hold	—	10°C	Hold

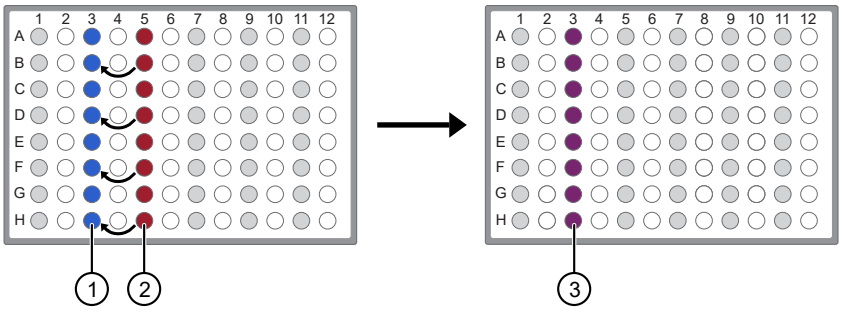
STOPPING POINT Target amplification reactions may be stored at 10°C overnight on the thermal cycler. For longer periods, store at –20°C.

Combine target amplification reactions

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

1. Remove the plate from the thermal cycler.
2. Centrifuge briefly to collect the contents, then carefully remove the plate seal.
3. For each sample, combine both 10-µL 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.



① Pool 1
② Pool 2
③ Combined pools

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

Partially digest the amplicons

IMPORTANT! Keep the 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 sample. The total volume is ~22 µL.
3. Seal the 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 minutes
55°C	20 minutes
60°C	20 minutes
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 chip, you *must* ligate a different barcode adapter to each library. DNA and RNA libraries from the same sample also require different barcodes.

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

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

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

Combine and dilute adapters—Ion Xpress™ Barcode Adapters only

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" on page 18.

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

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

Store diluted adapters at –20°C.

Perform the ligation reaction

1. If there is visible precipitate in the Switch Solution or the tube cap after thawing, vortex or pipet up and down at room temperature to resuspend before pipetting.
2. Briefly centrifuge the plate to collect the contents.
3. Carefully remove the plate seal, then add the following components in the order that is listed to each well containing digested amplicons. If preparing multiple non-barcoded libraries, a master mix of Switch Solution and adapters can be combined before addition.

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

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

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

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

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

STOPPING POINT Samples can be stored for up to 24 hours at 10°C on the thermal cycler. For longer periods, store at –20°C.

Purify the library

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

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

1. Prepare 70% ethanol (350 µL × number of samples) fresh daily.
2. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
3. 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.

4. Incubate the mixture for 5 minutes at room temperature.
5. 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.
6. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet to wash the beads. Carefully remove, then discard the supernatant without disturbing the pellet.

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

7. Repeat step 6 for a second wash.

8. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 2–5 minutes. Do not overdry.

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

Proceed immediately to "Elute the library". Alternatively, quantify the libraries using a Qubit™ Fluorometer, see .

Elute the library

1. Remove the plate with purified libraries from the plate magnet, then **add 50 µL of Low TE** to the pellet to disperse the beads.
2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
3. Incubate at room temperature for at least 2 minutes.
4. Place the plate on the magnet for at least 2 minutes.

   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 500-fold dilution for quantification. Remove 2 µL of supernatant, containing the library, then combine with 998 µL of Nuclease-free Water.

Quantify library by qPCR and calculate the dilution factor

Determine the concentration of each Oncomine™ Tumor Mutation Load Assay library by qPCR with the Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802). Analyze each sample, standard, and negative control in duplicate reactions. Libraries that have not undergone a second round of amplification typically have yields of 100–500 pM. However, yield is not indicative of library quality. After quantification, determine the dilution factor that results in a concentration of ~50 pM.

Note: Alternatively, quantify the libraries using a Qubit™ Fluorometer, see Appendix B, "Quantify the amplified library with the Qubit™ Fluorometer" for more information.

1. Prepare three 10-fold serial dilutions of the *E. coli* DH10B Ion Control Library (~68 pM; from the Ion Library TaqMan™ Quantitation Kit) at 6.8 pM, 0.68 pM, and 0.068 pM. Mark these tubes as standards, then use these concentrations in the qPCR instrument software.

Standard	Control Library	Nucease-free water[1]	Dilution factor	Concentration
1	5 µL undiluted Control Library	45 µL	1:10	6.8 pM
2	5 µL Std 1	45 µL	1:100	0.68 pM
3	5 µL Std 2	45 µL	1:1000	0.068 pM

[1] Not DEPC-treated.

Note: When you program the qPCR instrument, enter the concentration of each standard in the "Amount" field.

2. Calculate, then prepare the required volume of PCR master mix for duplicate reactions of each library sample, standard, and NTC using the following table. Include a 5–10% overage to accommodate pipetting errors.

Component	Volume per reaction (96-well plate)
2X TaqMan™ Master Mix	10 µL
20X Ion TaqMan™ Assay	1 µL
Total	**11 µL**

3. In an Optical PCR 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)
PCR Master Mix	11 µL
1:500 dilution of the sample[1]	9 µL

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

4. Program the real-time instrument as described in the following table.
   a. Enter the concentrations of the control library standards.
   b. Select ROX™ Reference Dye as the passive reference dye.

c. Select a reaction volume of 20 µL.

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

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

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

5. Following qPCR, calculate the average concentration of the undiluted Oncomine™ Tumor Mutation Load Assay library by multiplying the determined concentration × 500.

6. Based on the calculated library concentration, determine the dilution that results in a concentration of 50 pM for Ion Chef™ template preparation.
   - 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 about 50 pM.

7. Store undiluted libraries, or proceed to “Combine libraries for equal depth of coverage” and template preparation.

STOPPING POINT Undiluted 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.

Combine libraries for equal depth of coverage

Multiple barcoded Oncomine™ Tumor Mutation Load Assay libraries can be sequenced on a single chip by combining equal volumes of each barcoded library before template preparation.

IMPORTANT! You can prepare barcoded libraries from different samples using IonCode™ or Ion Xpress™ barcode adapters. However, do NOT combine libraries that are prepared with IonCode™ barcode adapters with libraries that are prepared with Ion Xpress™ barcode adapters.

We recommend combining up to a maximum of 8 libraries (Ion 540™ Chip), or 16 libraries (Ion 550™ Chip) when determining the Mutation Load only. For tumor profiling as well as the Mutation Load calculation we recommend combining up to 4 libraries (Ion 540™ Chip), or 6 libraries (Ion 550™ Chip), as described below.

Ion sequencing chip	Number of libraries	Volume to combine (X)[1]
Ion 540™ Chip	2	13.5 µL
	4	**6.8 µL**
	8	3.4 µL
Ion 550™ Chip	2	13.5 µL
	6	**4.5 µL**
	16	2.0 µL

[1] Provides sufficient volume of combined libraries for 1 templating reaction plus overage to accommodate pipetting error.

Note: Due to coverage variability in FFPE samples that may be due to sample quality, increased sequencing depth may be necessary to achieve sufficient coverage. Increased sequencing depth can be achieved by reducing the number of samples per chip.

To prepare a combined library:

1. Dilute all individual barcoded libraries to 50-pM concentration.
2. Combine X-µL of each library in a single 1.5-mL Eppendorf™ LoBind™ tube.
3. After adding the last library, pipet up and down 5 times to mix, then centrifuge briefly to collect in the bottom of the tube.

   IMPORTANT! Do not dilute the combined library further before templating, use at 50-pM concentration.
4. Proceed to templating and sequencing.

STOPPING POINT Combined libraries can be stored at 4–8°C for up to 48 hours before use.

Guidelines for templating and sequencing

Proceed to template preparation and sequencing using the following kits.

Template System	Sequencer	Kit	User Guide
Ion Chef™	Ion GeneStudio™ S5 System	Ion 540™ Kit – Chef (Cat. Nos. A27759, A30011)	*Ion 540™ Kit – Chef User Guide* (Pub. No. MAN0010851)
		Ion 550™ Kit – Chef (Cat. No. A34541)	*Ion 550™ Kit – Chef User Guide* (Pub. No. MAN0017275)

IMPORTANT! When templating the combined libraries, use at 50 pM concentration. Do NOT dilute further before loading the Ion Chef™ Instrument.

To create a specific Run Plan for use in templating and sequencing see “Create a custom Planned Run template” on page 26. See the appropriate user guide listed in the table for more information.

4 Create an assay-specific Planned Run

IMPORTANT!

- We recommend that you update to the latest available versions of Torrent Suite™ and Ion Reporter™ Software to enable the data analysis and reporting options that are described in this chapter. Torrent Suite™ Software 5.6 or later is required to access the preinstalled Planned Run templates.
- These kits are compatible with Torrent Suite™ Software 5.6 or later and Ion Reporter™ Software 5.10 or later. Before proceeding, check for updates to the Torrent Suite™, Ion Reporter™, and Ion Chef™ System software, and install the updates if available.
- Sequencing on an Ion 550™ Chip requires Torrent Suite™ Software 5.8 or later.
- Install the IonReporterUploader plugin for Torrent Suite™ Software. The plugin version that is installed must match the Ion Reporter™ Software version. See "Install the IonReporter Uploader plugin on your Torrent Server" on page 46 for more information.

About Run Plans

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

In Torrent Suite™ Software 5.6 or later, the primary Planned Run template to be used with the Oncomine™ Tumor Mutation Load Assay is the **Oncomine™ Tumor Mutation Load** template.

Application	Torrent Suite™ Software template	Description
Mutation Load	Oncomine™ Tumor Mutation Load	DNA-only planned run template

Create a custom Planned Run template

IMPORTANT!

We recommend setting up a customized Planned Run template for reuse when the same conditions will be used for multiple runs. For more information about creating Planned Runs manually or from the generic application template, see the online help available in the Torrent Suite™ Software.

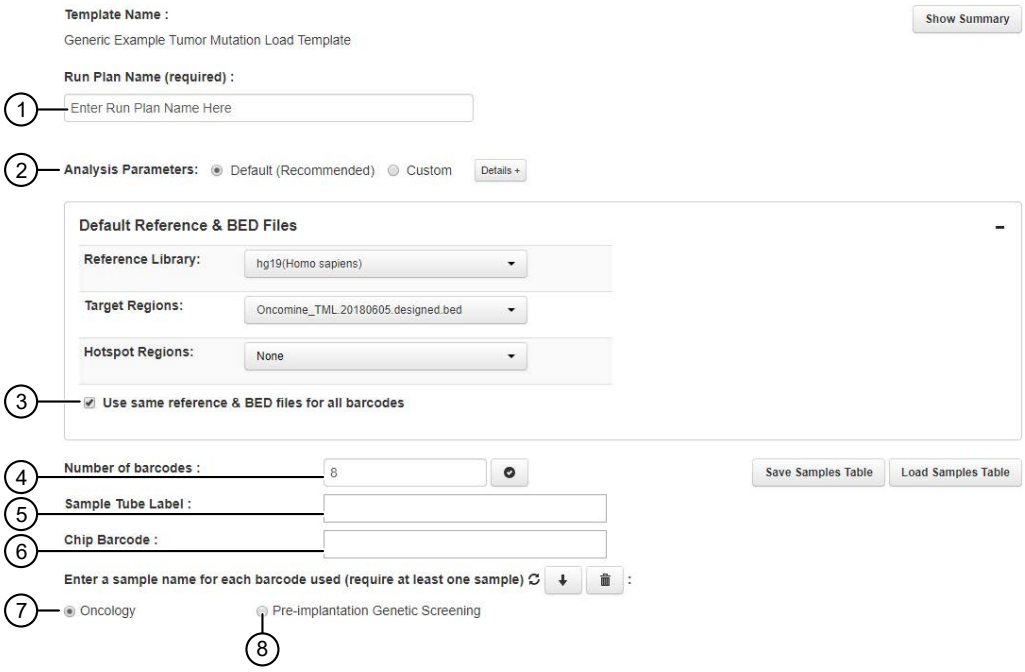
1. Sign in to the Torrent Suite™ Software.
2. In the **Plan** tab, click **Templates**, then click **Mutation Load** in the left navigation menu.

The customized template is now available in the **Templates** screen of the **Mutation Load** Research Application. If you set your customized template to favorites, it also appears in the **Favorites** list.

Create a Planned Run from a template

1. Sign in to the Torrent Suite™ Software.
2. In the **Plan** tab, click **Templates**, then click **Mutation Load** in the left navigation menu.
3. In the **Mutation Load** list, click on your customized Planned Run template name or the preinstalled system template. Alternatively, in the row of the template click ⚙ ▸ **Plan Run**.
   The **Create Plan** workflow bar opens to the **Plan** step.
4. Enter or select the following information. Row numbers in the table correspond to the callouts in the following figure.

Callout	Field	Action
1	Run Plan Name	Enter a Run Plan name.
2	Analysis Parameters	Ensure the Default (Recommended) radio button is selected.
3	Use same reference & BED files for all barcodes	Ensure that the checkbox is selected.
4	Number of barcodes	Enter the number of barcodes that will be used in this run, then click the ✔ button to the right of this field.
5	Sample Tube Label	Enter or scan the barcode of the Ion Chef™ sample tube that will be used in the run.
6	Chip ID	No entry required.
7	Oncology	Ensure that the radio button is selected.
8	Pre-implantation Genetic Screening	Ensure that the radio button is unselected.



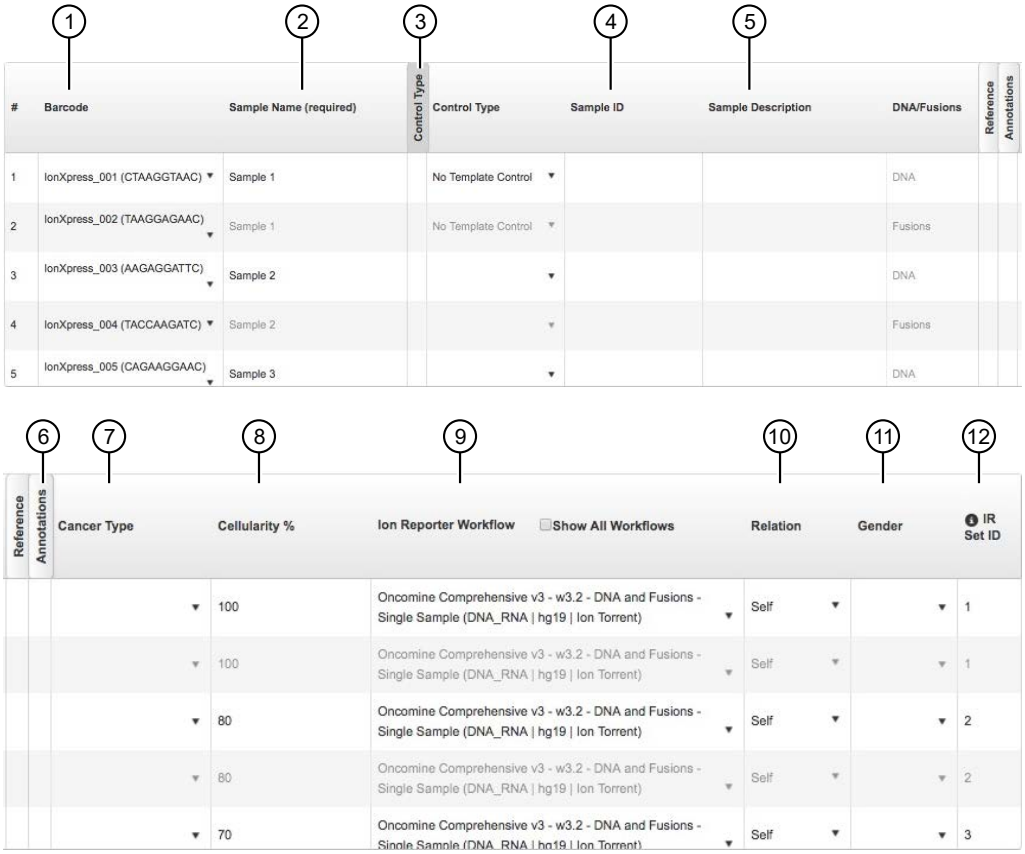
5. Enter sample information. Row numbers in the table correspond to the callouts in the following figure.

Callout	Field[1]	Action
1	Barcode	For each sample select the **Barcode** that will identify it from the dropdown list.
2	Sample Name	Accept the auto-populated sample names or click in a field, then enter a unique sample name. We recommend sample names (either auto-populated or user defined) be unique even between runs.
3	Control Type (expanded)	Select No Template Control from the dropdown list to designate a sample as a no template control.
4	Sample ID	*(Optional)* Click in the field, then enter a sample ID.
5	Sample Description	*(Optional)* Click in the field, then enter a sample description.
6	Annotations (expanded)	Click to reveal Cancer Type and Cellularity %.
7	Cancer Type	*(Optional)* Click in the field, then select from the dropdown list. Click to copy the entry to all the rows.
8	Cellularity %	*(Optional)* Click in the field, then enter a value. Click to copy the entry to all the rows.
9	Ion Reporter Workflow	Ensure the correct workflow is selected.
10	Relation	*(Optional)* Click in the field, then ensure the correct value is auto-populated. Select from the dropdown list to change.

(continued)

Callout	Field[1]	Action
11	Gender	*(Optional)* Click in the field, then select from the dropdown list. Click to copy the entry to all the rows.
12	IR Set ID	The IR Set ID links individual samples for analysis. Ensure the auto-populated value is unique to each sample. Select from the dropdown list to change.

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



6. Click **Plan Run**.

The run is listed in the **Planned Run List** 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 Mutation load analysis

Review sequencing run results

If when creating your Planned Run you selected the data upload option to **Review results after run completion, then upload to Ion Reporter** you can review your sequencing results in the Torrent Suite™ Software by running the coverageAnalysis plugin. The coverageAnalysis plugin generates a report that describes the level of sequence coverage produced for targeted genomic regions.

1. After the sequencing run is complete, in the **Data** tab, click **Completed Runs & Reports**.
2. In the list of runs, find the run of interest, then click the link in the **Report Name** column.
3. In the left navigation menu, click **coverageAnalysis** to view the plugin summary.

4. Review the results in the **Mean Depth**, and **Uniformity** columns to ensure that the thresholds have been met for each barcoded library.

Column	Description	Threshold
Mean Depth	Reports mean read depth across all targeted regions.	≥1100[1]
Uniformity	The percentage of bases in all targeted regions (or whole-genome) covered by at least 20% of the average base coverage depth reads. (Cumulative coverage is linearly interpolated between nearest integer base read depths.)	≥80%

[1] Provides sufficient read depth for variant calling at ≥5% allele frequency. If only determining the Mutation Load, ≥300 mean read depth is sufficient.

Note: Due to coverage variability in FFPE samples that may be due to sample quality, increased sequencing depth may be necessary to achieve sufficient coverage. Increased sequencing depth can be achieved by reducing the number of samples per chip.

Ion Reporter™ analysis workflows

If you selected the appropriate Ion Reporter™ workflow when setting up your Planned Run in Torrent Suite™ Software, automated analysis has already been performed and you can view the Mutation Load analysis results in Ion Reporter™ Software. For instructions on manually launching an analysis, see page 52.

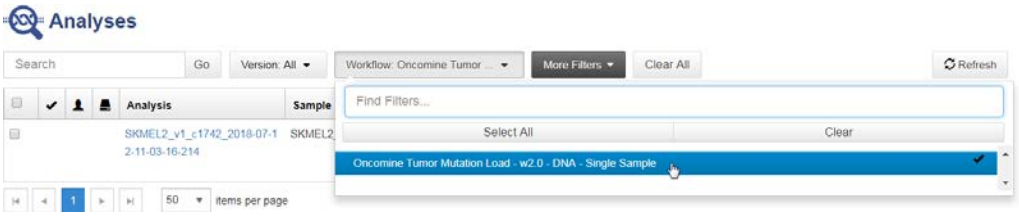
Note: Microsoft™ Excel™, or other spreadsheet tool, is required for viewing VCF, CSV, and TSV files.

Analysis Workflow	Description
Oncomine™ Tumor Mutation Load - w2.0 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs and INDELs) from targeted DNA libraries from the Oncomine™ Tumor Mutation Load Assay run on the Ion 540™ or Ion 550™ Chip. Computes tumor mutational burden (TMB) from a single sample using the Mutation Load (Somatic Mutations) filter chain and TMB Algorithm Version 2.5. Released with: Ion Reporter™ Software 5.10. Workflow version: 2.0.
Oncomine™ Tumor Mutation Load - w1.0 - DNA - Single Sample	Compute tumor mutation load from single sample. Released with: Ion Reporter Software 5.6. Workflow Version: 1.0.

View results

Ion Reporter™ Software analyses are performed automatically on uploading of the data files from the Torrent Suite™ Software. To view the results and calculated Mutation Load (mutations/Mb):

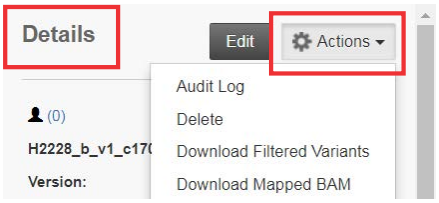
1. Sign in to the Ion Reporter™ Software.
2. Click the **Analyses** tab.
3. In the **Workflow** dropdown list, select **Oncomine™ Tumor Mutation Load - w2.0 - DNA - Single Sample** to limit the list of results to analyses using this workflow.



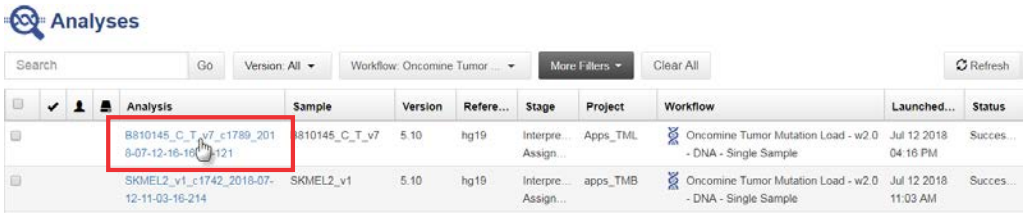
You can further refine the list of analyses by applying **More Filters**, or clicking column headers. The **Analyses** table automatically filters the content appropriate to your selection.

4. Click within a row (but not on the sample data set hyperlink) to view the **Details** of the analysis.

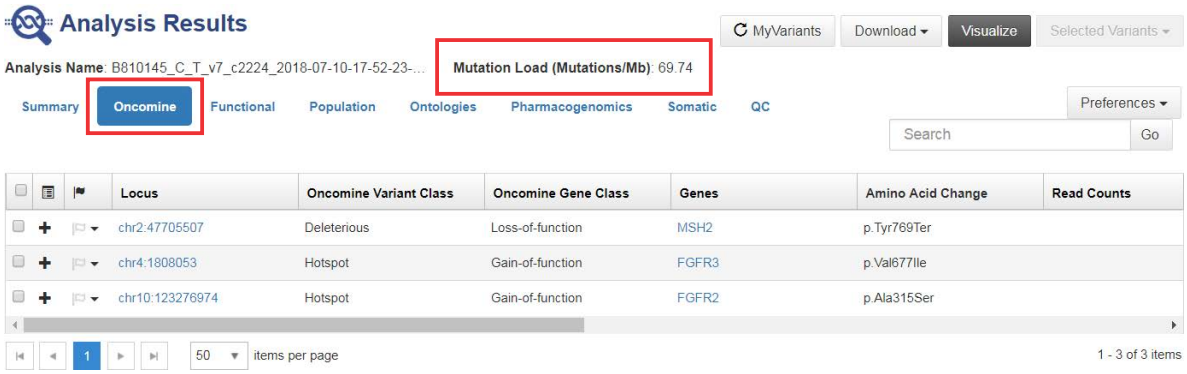
   In the **Details** pane you can view **Workflow Details**, and access the **Actions** dropdown list.



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



The mutation load is displayed at the top of the results page in mutations per Mb. The **Analysis Results** page opens to the **Oncomine** tab displaying only Oncomine™ annotated variants that are known to be cancer drivers.



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

   a. In the **Filter Options** pane, select the desired **Filter Chain**.

   For example, selecting the **Mutation Load (Somatic Mutations)** filter chain displays all somatic mutations used in the Mutation Load analysis.

Note:

- The default **Filter Chain** is **Oncomine Variants (5.10)** which limits the results displayed to cancer driver variants only. Each variant called must meet all the conditions of the filter chain to have been filtered-in. For more information, see “Oncomine™ Tumor Mutation Load Assay filter chain criteria” on page 48 .
- Select **No Filter** to view all the variant calls attempted by the variant caller. To view only the variant calls used in calculating the Mutation Load select **Mutation Load (Somatic Mutations) (5.10)**.
- Saving the analysis using a filter chain other than **Oncomine Variants (5.10)** changes the variant calls that are saved in the VCF file and can affect downstream workflows, such as with Oncomine™ Reporter Software.

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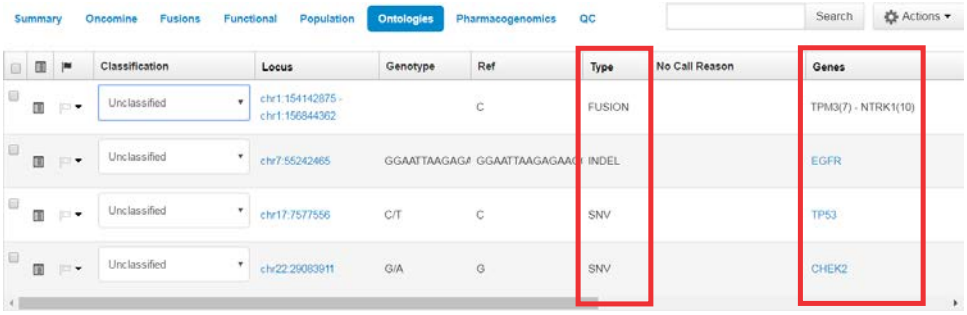
b. In the **Oncomine** tab, click the column headers to sort the list of variants by **Oncomine Variant Class** or **Oncomine Gene Class**.

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



7. To download a results file.

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

b. In the **Home** tab, click **Notifications**, then click next to the file name. Alternatively, select one or more rows, then click **Download**.

The software generates a ZIP file with 4 folders: RESULTS, QC, Variants, and Workflow_Settings.

Folder	Contents of folder
RESULTS	Contains: • TSV files: `filtered_variants.tsv` and `somatic_variants.tsv`. Both contain somatic mutations, or the mutations that are left after applying Mutation Load filter chain, from both exonic and non-exonic regions of the panel. The sum of variants in both files equals the "Total Somatic Variants Count" in the `statistic.txt` file. Only the missense and nonsense SNVs, and frameshift and non-frameshift INDELs of these files, are included in the TMB calculation. • PDF of the Mutation Load analysis report. • File named `statistic.txt`, which contains mutation load statistics. **Note:** The Variant Details TSV file can be downloaded separately if you prefer. For more information see “Visualize Mutation Load analysis results” on page 35. Also contains PNG image files: • allele frequency distribution of germ-line and somatic variants • allele frequency distribution of only somatic variants • bar plot of signature type and context of somatic mutations • pie chart of substitution type of somatic mutations • pie chart of signature pattern of somatic mutations
QC	Contains a PDF of the QC report, and a folder containing coverage statistics files.

(continued)

Folder	Contents of folder
Variants	• Contains intermediate and Oncomine™ annotated .VCF files, which are used by Oncomine™ Reporter Software. For more information, see the *Oncomine™ Reporter 3.0 User Guide* (Pub. No. MAN0017300). • TSV files that contain Oncomine™-filtered and all somatic variants.
Workflow_Settings	Contains folders with: • 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 workflow settings.

Generate an Analysis Results Report

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

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

To view an example Analysis Report, see page 57. A separate Mutation Load Analysis Report including graphical representations of the data can be downloaded from the Analysis Visualization page, for more information see “Visualize Mutation Load analysis results” on page 35.

View the Mutation Load statistics

The Mutation Load value is calculated automatically when the Oncomine™ Tumor Mutation Load - w2.0 - DNA - Single Sample workflow is run. To view more details of the analysis (for example, the TMB algorithm version used), and how the Mutation Load is calculated.

1. Download, then extract a results file to your hard drive.
2. In the **RESULTS** folder, open the `statistic.txt` file in a compatible text editor. The Mutation Load and the data that are used to calculate its value are listed.

```
Total Variant Count=1,050
Total Somatic Variant Count=110
COSMIC Annotated Somatic Variants=16
Total Bases with Sufficient Coverage=1651662
Average Coverage=697.0
Total Exonic Bases with Sufficient Coverage=1190845
Deamination=31
Calibration Intercept (for Mutation Load greater than equal to 25)=0
Calibration Slope (for Mutation Load greater than equal to 25)=1.4637
Nonsynonymous Somatic Mutations=60
Nonsynonymous Somatic SNVs=59
Nonsynonymous Somatic Indels=1
Mutation Load (Mutations/Mb)=62.16
Released with Ion Reporter 5.10. TMB Algorithm Version=2.5
Variant count=110
Nonsynonymous=60
Synonymous=19
```

Values used in calculating the Mutation Load (red box) are highlighted (yellow).

Mutation Load calculation

$$\text{precalibration Mutation Load} = \frac{\text{Nonsynonymous Somatic Mutations} \times 10^6}{\text{Total Exonic Bases with Sufficient Coverage}}$$

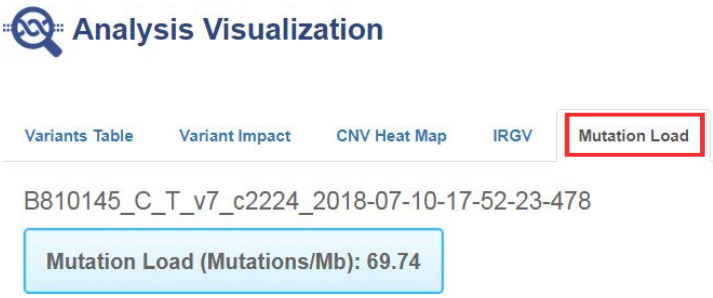
IF, precalibration Mutation Load ≥25, THEN Mutation Load = (precalibration Mutation Load – 25) x Calibration Slope + 25

IF, precalibration Mutation Load <25, THEN no calibration is required,
Mutation Load = precalibration Mutation Load x 1.0

Visualize Mutation Load analysis results

Visualizing the Ion Reporter™ Software analyses allows you to view a single sample result or multiple sample results simultaneously. Individual sample results can be visualized from either the **Analyses** table or **Analysis Results** screen.

- To visualize sample results individually from the **Analyses** table.
  a. In the **Analyses** table select a sample result row, then click **Visualize**. Alternatively, click **Actions** ▸ **Visualize**.



  The Analysis Visualization screen opens to the Mutation Load tab with the tumor mutation load (Mutations/Mb) displayed at the top.

  b. Select either the **Sample Results** or **Sample QC** tab, then select the graphical representation of the data from the **Views** dropdown list.

  c. Click **Download Report**.
  A 2-page PDF Mutation Load Analysis Report of the results including copies of the graphs is automatically downloaded, see page 58.

  d. In the lower right corner, click **Download Variant Details TSV**.
  A tab separated values list of each variant detected is automatically downloaded. Open the file with a TSV compatible viewer to see details of each individual variant that contributed to the Mutation Load count.

- To visualize sample results individually from the **Analysis Results** page.
  a. In the **Analyses** table, click a sample result hyperlink in the **Analysis** column to open the **Analysis Results**.

  b. Click **Visualize**.

  c. Select either the **Sample Results** or **Sample QC** tab, then select the graphical representation of the data from the **Views** dropdown list.

  d. Click **Download Report**.
  A 2-page PDF Mutation Load Analysis Report of the results including copies of the graphs is automatically downloaded, see page 58.

e. In the lower right corner, click **Download Variant Details TSV**.
   A tab separated values list of each variant detected is automatically downloaded. Open the file with a TSV compatible viewer to see details of each individual variant that contributed to the Mutation Load count.

- To visualize multiple sample results simultaneously.
  a. In the **Analyses** table, select the checkbox next to each sample result you want to visualize together, then click **Visualize**. Alternatively, in the **Selected Analyses** pane, click **Actions** ▸ **Visualize**.

  b. Select either the **Sample Results** or **Sample QC** tab, then select the graphical representation of the data from the **Views** dropdown list.

  c. Click **Download Report** for each individual sample result.
     A 2-page PDF Mutation Load Analysis Report of the results including copies of the graphs is automatically downloaded, see page 58.

  d. In the lower right corner, click **Download Variant Details TSV**.
     A tab separated values list of each variant detected is automatically downloaded. Open the file with a TSV compatible viewer to see details of each individual variant that contributed to the Mutation Load count.

Sample Results tab

The Oncomine™ Tumor Mutation Load Assay results are represented graphically. Select the Sample Results to view from the **Views** dropdown list.

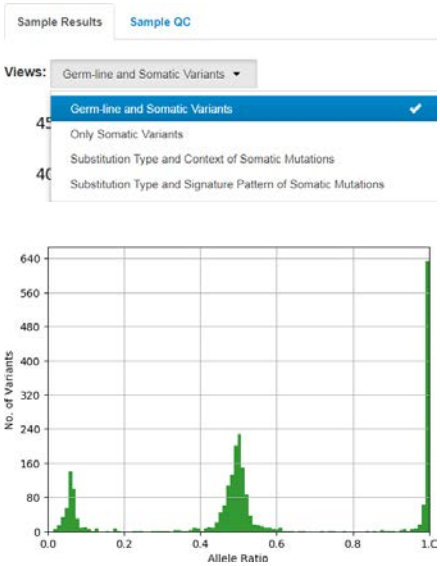


Figure 1 Allele ratio of Germline and Somatic Variants

A histogram showing 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. Value is reported in the `statistic.txt` file as **Total Variant Count**, see"View the Mutation Load statistics" on page 34.

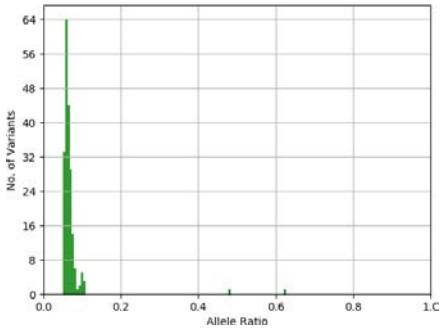


Figure 2 Allele ratio of Only Somatic Variants

A histogram showing frequency distribution of allele ratio for only somatic mutations as determined by the Mutation Load (Somatic Mutations) filter chain. Listed below the figure are:

- The number of **Total Somatic Variants** (Value is reported in the `statistic.txt` file as **Total Somatic Variant Count**, see"View the Mutation Load statistics" on page 34).
- A break down of the number of mutations determined to be nonsynonymous (detrimental) and synonymous (non-detrimental) as annotated by Ion Reporter™ Software. Values are reported in the `statistic.txt` file, see"View the Mutation Load statistics" on page 34.
- The number of detected somatic variants found in the COSMIC database. Value is reported in the `statistic.txt` file as **COSMIC Annotated Somatic Variants**, see"View the Mutation Load statistics" on page 34.

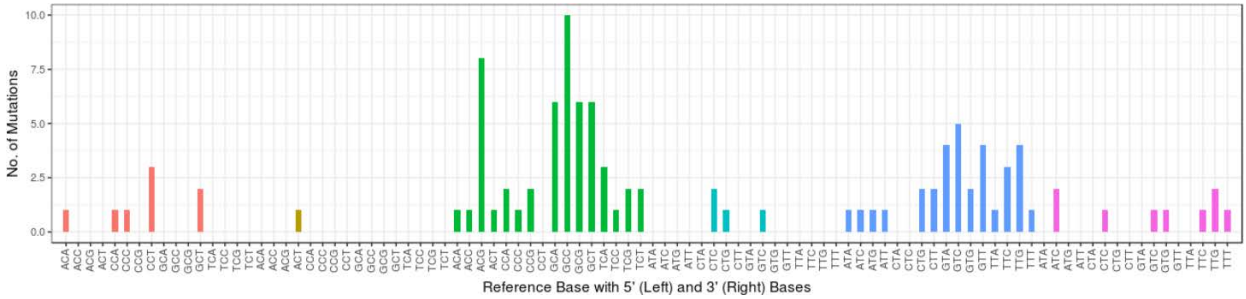


Figure 3 Substitution Type and Context of Somatic Mutations

C>A
C>G
C>T
T>A
T>C
T>G

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, there are 96 possible mutation types 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.

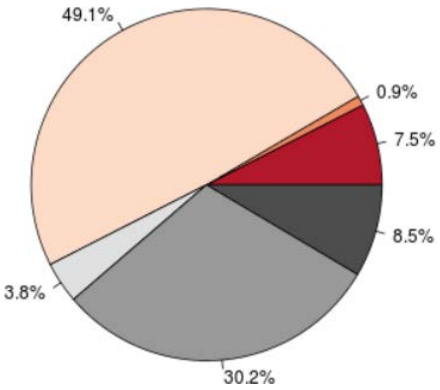


Figure 4 Substitution type of Somatic Mutations

C>T
C>G
C>A
T>G
T>C
T>A

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.

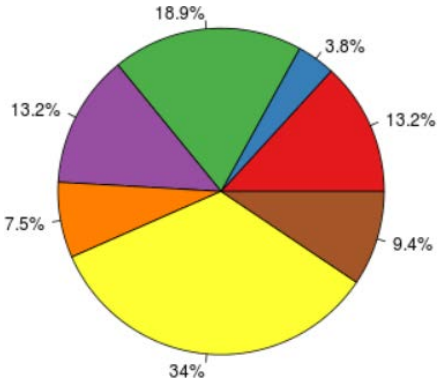


Figure 5 Signature Pattern of Somatic Mutations

C>T at [AG]CG
C>T at [CT]CG
C>T at NCC,CC[ACT],TC[ACT]
C>T at other sites
C>A
T>A and T>C
Rest

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

In Figure 5, 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.

- High C>T at CpC, CpC, TpC, T>A, and T>C is consistent with UV damage (Hayward, 2017). (Blue + Green + Yellow)
- High C>T at CpG is consistent with spontaneous deamination of 5-methylcytosine (Alexandrov, 2013). (Red + Blue)
- High C>A is consistent with smoking damage (Alexandrov, 2016). (Orange)
- High C>T (site independent) is consistent with FFPE processing (Wong, 2014). (Green + Purple)

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

Sample QC tab

The sample QC tab lists the:

QC metric	Definition
Average Coverage	The average read depth across covered nucleotide bases. Value is reported in the `statistic.txt` file as **Average Coverage**, see "View the Mutation Load statistics" on page 34.
Number of Calls	The number of variants identified in the sample. Value is reported in the `statistic.txt` file as **Total Variant Count**, see "View the Mutation Load statistics" on page 34.
Estimated SNP proportion consistent with Deamination (mainly FFPE)	The proportion of identified mutations that are likely the result of FFPE sample deamination during processing. Value is reported in the `statistic.txt` file as **Deamination**, see "View the Mutation Load statistics" on page 34.

A

Troubleshooting

Tips

- Arrange samples in alternating columns on the plate for easier pipetting with multichannel pipettes during purification with the DynaMag™ Side Magnet.
- Plate seals can be firmly applied using the applicator in the MicroAmp™ Optical Adhesive Film Kit. Plate seals can be removed with much less effort when hot. Try removing seals right after taking the plate out of the thermal cycler.
- If library yield is below 50 pM, libraries can still be sequenced by using a proportionally larger volume into a combined library or into template preparation.
- If the unamplified library yield is below 50 pM, libraries can be rescued with library amplification. Combine 25 µL of unamplified library with 72 µL of 1X Library Amp Mix and 4 µL of 25X Library Amp Primers. Perform 5–10 library amplification cycles using the following cycling conditions.

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

- 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.

Observation	Possible cause	Recommended action
Sample resulted in a high deamination score	• Old FFPE sample. • Improperly fixed FFPE sample. • Improper sample storage conditions.	Edit the Ion Reporter™ workflow, then reanalyze the data at a higher minimum allele frequency. For more information, see "Customize the Ion Reporter™ workflow" on page 53.
		Treat the FFPE sample DNA with Uracil DNA Glycosylase (UDG), then resequence. For more information, see "Remove deaminated bases from FFPE DNA" on page 55.
Library concentration is low–general **Details:** (Library concentration is NOT indicative of quality.)	Sample DNA was mis-quantified.	Requantify sample DNA using the TaqMan™ RNase P Detection Reagents Kit.
	Sample DNA quality was low.	Add more DNA or increase target amplification cycles.
	PCR, digestion, or ligation was inefficient.	Ensure proper dispensing and mixing of viscous components at each step.

Observation	Possible cause	Recommended action
Library concentration is low–general **Details:** (Library concentration is NOT indicative of quality.) *(continued)*	Residual ethanol in the sample DNA inhibited target amplification.	Incubate uncapped tube in hood for 1 hour.
		Speed-vac tube at room temperature for 5 minutes.
	Residual ethanol from AMPure™ purification inhibited library amplification.	Carefully remove all drops of ethanol before library amplification, then centrifuge plate, if needed.
	AMPure™ XP beads were over-dried.	Do not dry the AMPure™ XP beads more than 5 minutes.
Percentage of polyclonal ISPs is high (>40%)	Library input was too high.	Decrease amount of library added to the template preparation reaction by 50%.
	Library was mis-quantified.	Ensure that library was quantified accurately.
	Other.	Check the appropriate template preparation user guide for more information.
Low quality ISPs are present at high percentage (>15%)	Library input was too low.	Double the volume of library used in template preparation.
		Use a fresh dilution of library prepared in a low-bind tube.
	Other.	Check the appropriate template preparation user guide for more information.

B

Quantify the amplified library with the Qubit™ Fluorometer

Oncomine™ Tumor Mutation Load Assay libraries must be amplified before quantification to enrich amplifiable material and obtain sufficient material for accurate quantification. Amplify the library using 1X Library Amp Mix, then purify. Quantify the library using the Qubit™ 4 Fluorometer. Amplified libraries typically have yields of 2,000–10,000 pM. Yield is not indicative of library quality. After quantification, determine the dilution factor that results in a concentration of ~50 pM, which is appropriate for template preparation using an Ion template kit.

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

Amplify the library

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

   Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
3. Place the plate back on the magnet for at least 2 minutes, then carefully transfer ~50 µL of supernatant from each well to a new well or a new plate without disturbing the pellet.
4. Seal the plate with MicroAmp™ Clear Adhesive Film, place a MicroAmp™ Optical Film Compression Pad on the plate, load in the thermal cycler, then run the following program.

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

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

Purify the amplified library

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

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

IMPORTANT!

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

First-round purification

1. Tap the plate gently on a hard flat surface, or briefly centrifuge to collect the contents, then remove the plate seal.
2. Add 25 µL (0.5X sample volume) of Agencourt™ AMPure™ XP Reagent to each plate well containing ~50 µL of sample. Mix the bead suspension with the DNA thoroughly by pipetting up and down 5 times.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a magnet such as the DynaMag™–96 Side Magnet for at least 5 minutes, or until the solution is clear.
5. Carefully transfer the supernatant from each well to a new well of the 96-well PCR plate without disturbing the pellet.

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

Second-round purification

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

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

4. **Add 150 µL of freshly prepared 70% ethanol** to each well, then move the plate side to side in the magnet to wash the beads. Remove, then discard the supernatant without disturbing the pellet.

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

5. Repeat step 4 for a second wash.

6. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 2–5 minutes. **Do not overdry.**

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

8. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge to collect droplets. Alternatively, mix by setting a pipettor to 40 µL, then pipet the mixture up and down at least 5 times before sealing the plate.

9. Incubate at room temperature for at least 2 minutes.

10. Place the plate in the magnet for at least 2 minutes, then analyze an aliquot of the supernatant as described in:

    - "Qubit™ Fluorometer—Quantify the library and calculate the dilution factor" on page 45 or
    - "Quantify library by qPCR and calculate the dilution factor" on page 21

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

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

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

1. Determine the amplified library concentration.
   a. Make a 1:200 working dilution of Qubit™ dsDNA HS Reagent using the Qubit™ dsDNA HS Buffer.
   b. Combine 10 µL of the amplified Ion AmpliSeq™ library with 190 µL of dye reagent, mix well, then incubate for at least 2 minutes.
   c. Prepare each Qubit™ standard as directed in the user guide.
   d. Measure the concentration on the Qubit™ Fluorometer.
   e. *(Qubit™ 2.0 Fluorometer only)* Calculate the concentration of the undiluted library by multiplying by 20. Alternatively, use the **Calculate Stock Conc.** feature on your instrument.
2. Based on the calculated library concentration, determine the dilution that results in a concentration of 40–60 pM.

Average amplicon size	Concentration in ng/mL (40–60 pM)
175 bp	5.5
225 bp	7.5

   For example, with a FFPE-compatible 125–175 bp design (average 225 bp with adapters):
   - The library concentration is 450 ng/mL.
   - The dilution factor is 450 ng/mL divided by 7.5 ng/mL = 60.
   - Therefore, 5 µL of library that is mixed with 295 µL of Low TE (1:60 dilution) yields approximately 7.5 ng/mL (~50 pM).
3. Dilute the library to ~50 pM as described, combine, then proceed to template preparation, or store libraries as described in “Store libraries” on page 45.

Store libraries

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

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

C Supplemental information

Torrent Suite™ Software

Install the IonReporter Uploader plugin on your Torrent Server

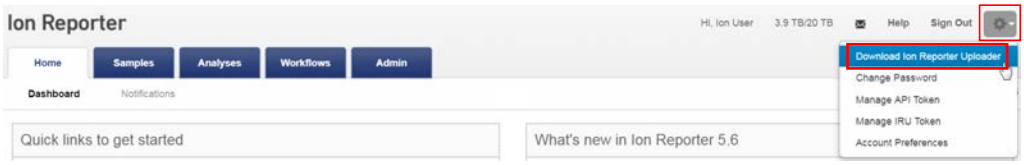
The IonReporterUploader 5.10 plugin is automatically installed on Torrent Server when you update to a new release.

Note: If your instrument server does not have internet access, you must download and install the latest file named `IonReporterUploader_<version>.deb` from **http://iru.ionreporter.thermofisher.com/** before proceeding.

To reinstall or update the IonReporterUploader 5.10 plugin for Torrent Suite™ Software 5.2 or later.

Note: An administrative **ionadmin** account is not required for this procedure.

1. Sign in to Ion Reporter™ Software, then click ⚙ **(Settings)** ▸ **Download Ion Reporter Uploader**.



2. Click the filename **IonReporterUploader.zip**, then download the file to your local machine.
3. Sign in to Torrent Suite™ Software, then click ⚙ **(Settings)** ▸ **Plugins**.
4. Click **Install or Upgrade Plugin**.

5. Click **Upload a Plugin file**, then browse to the **IonReporterUploader.zip** file. Click **Open**, click **Upload**, then click **Install**.

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.

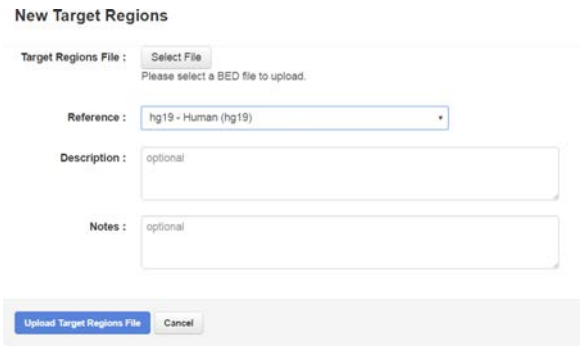
6. Click **Get Versions**, select **Ion Reporter 5.10 or later**, then click ✓**Add**.

Download and install BED files

Contact your field service representative to obtain the latest versions of Oncomine™ Tumor Mutation Load Assay BED files.

1. Extract the BED file containing ZIP file to a location of your choice.
2. Sign in to the Torrent Suite™ Software where you want to install the **Target Regions** BED file.
3. In the upper right of the screen, click ⚙ ▾ **(Settings)** ▸ **References**.
4. Upload the Target Regions panel BED file:
   a. In the left navigation menu, click **Target Regions**, then click **Add Target Regions**.
   b. Select **hg19 - Homo sapiens** from the **Reference** dropdown list.

c. Click **Select File**, then navigate to and select the Target Regions file: **Oncomine_TML.20180605.designed.bed**



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

The **Target Regions** BED file uploads to your Torrent Server and appears in the **Target Regions** dropdown list.

Ion Reporter™ Software

Oncomine™ Tumor Mutation Load Assay filter chain criteria

Variant Type	Oncomine™ Gene Class	Oncomine™ Variant Class	Annotation Criteria
Loss of Function Mutation	Loss-of-Function	Deleterious	• Positive mutation call • Variant's functional impact is frameshift block substitution, frameshift insertion, frameshift deletion, or nonsense • Variant occurs in a loss-of-function gene
Gain of Function Missense Hotspot Mutation	Gain-of-Function	Hotspot	• Positive mutation call • Variant's functional impact is missense • Variant occurs in a gain-of-function gene • Variant's transcript and codon position occur in predefined missense hotspot list
Loss of Function Missense Hotspot Mutation	Loss-of-Function	Hotspot	• Positive mutation call • Variant's functional impact is missense • Variant occurs in a loss-of-function gene • Variant's transcript and codon position occur in predefined missense hotspot list

(continued)

Variant Type	Oncomine™ Gene Class	Oncomine™ Variant Class	Annotation Criteria
Gain of Function In Frame Hotspot Mutation	Gain-of-Function	Hotspot	• Positive mutation call • Variant occurs in a gain-of-function gene • Variant's function, transcript, and coding syntax occur in predefined in-frame hotspot list
Loss of Function In Frame Hotspot Mutation	Loss-of-Function	Hotspot	• Positive mutation call • Variant occurs in a loss-of-function gene • Variant's function, transcript, and coding syntax occur in predefined in-frame hotspot list
Gain of Function Splice Site Hotspot Mutation	Gain-of-Function	Hotspot	• Positive mutation call • Variant occurs in a gain-of-function gene • Variant's transcript, location, and exon occur in predefined splice site hotspot list
Loss of Function Splice Site Hotspot Mutation	Loss-of-Function	Hotspot	• Positive mutation call • Variant occurs in a loss-of-function gene • Variant's transcript, location, and exon occur in predefined splice site hotspot list
Gain of Function Promoter Hotspot Mutations	Gain-of-Function	Hotspot	• Positive mutation call • Variant occurs in a gain-of-function gene • Variant's transcript, location, and coding syntax occur in predefined promoter hotspot list

Enable tumor mutational burden calculation in existing analysis workflows

Tumor mutational burden (TMB) is a calculation of somatic mutations per megabase (Mb). You can enable the calculation in any DNA – Single Sample, or DNA and Fusions – Single Sample analysis workflow. When enabled, tumor mutational burden and other data values are included in analysis results from that analysis workflow. To enable the tumor mutational burden calculation, copy and edit any predefined analysis workflow, or edit an existing custom analysis workflow that is not locked.

An analysis workflow that is enabled for tumor mutational burden calculates mutations per megabase (Mb) and adds graphics and other information about the mutations to Ion Reporter™ Software analysis results and visualizations.

Analysis workflows for use with the Oncomine™ Tumor Mutation Load Assay, such as Oncomine™ Tumor Mutation Load - w3.0 - DNA - Single Sample analysis workflow, are enabled for tumor mutational burden by default.

Note: Tumor mutational burden calculation is available in Ion Reporter™ Software 5.10 or later.

1. In the **Workflows** tab, click **Overview**.

2. In the list of analysis workflows, select the row for the DNA – Single Sample, or DNA and Fusions – Single Sample analysis workflow that you want to copy.
   The Details pane shows information about the selected workflow.

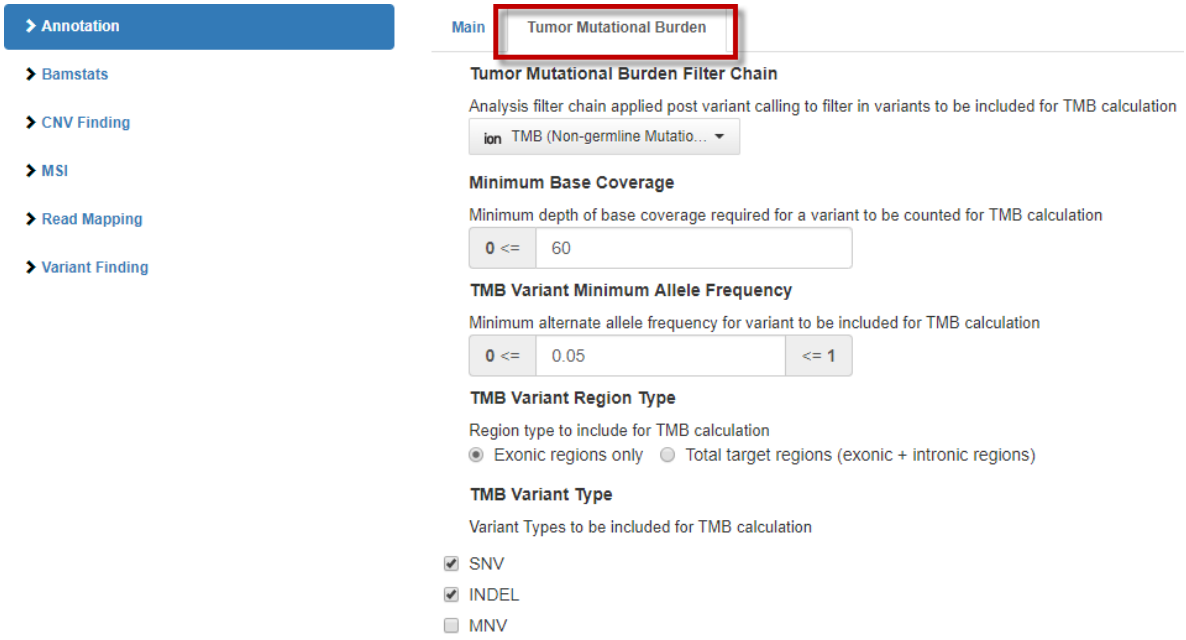
3. Click **(Actions)** ▸ **Copy**.
   The **Edit** workflow bar opens to the **Research Application** step with the **Research Application** and **Sample Groups** preselected. When you copy an analysis workflow template, you cannot change these settings.

4. Click **Next** multiple times to proceed to the **Parameters** step. Alternatively, click each step in the workflow bar to go to the parameter step.

5. To enable the tumor mutational burden calculation, ensure that the **Tumor Mutational Burden Filter Chain** parameter is set.
   a. In the **Parameters** step, under annotation, select the **Tumor Mutational Burden** tab.
   b. Find the **Tumor Mutational Burden Filter Chain** parameter, then change the value to **TMB (Non-germline Mutations**).

6. Change other tumor mutational burden parameters, if needed. For more information, see the Ion Reporter™ Software help system.

IMPORTANT! The parameter settings of an Oncomine™ Tumor Mutation Load are optimized. If you are using these parameters for another type of analysis workflow, change the parameters if needed. Contact your Field Bioinformatics Specialist (FSB) for assistance.



7. Click **Next**, then enter a **Workflow Name** and an optional **Description** for the analysis workflow.

8. Click **Save Workflow**.
   The custom analysis workflow is saved and is added to the **Workflows** table.

To ensure that the analysis workflow was saved, click the **Workflows** tab, then click **Overview**, and search for the analysis workflow name. To analyze samples with your new tumor mutational burden calculation enabled analysis workflow, see “Manually launch an analysis in Ion Reporter™” on page 52.

Manually launch an analysis in Ion Reporter™

1. Sign in to the Ion Reporter™ Software.
2. Launch the analysis.

From the…	Directions
Home tab	a. In the **Dashboard** screen, click **Launch analysis**. b. In the **Launch Analysis** screen, select **DNA** from the **Research Application** dropdown list. c. In the **Research Category** dropdown list, select **Oncology - ImmunoOncology**, then click in the **Oncomine™ Tumor Mutation Load - w2.0 - DNA - Single Sample** row. d. Click **Next**.
Analyses tab	a. Click **Launch Analysis**, then select **Manual** from the dropdown list. b. In the **Launch Analysis** screen, select **Mutation Load** from the **Research Application** dropdown list. c. Click in the **Oncomine™ Tumor Mutation Load - w2.0 - DNA - Single Sample** row, then click **Next**.
Workflows tab	a. Select **Mutation Load** from the **Research Application** dropdown list. b. Click in the **Oncomine™ Tumor Mutation Load - w2.0 - DNA - Single Sample** row, then select **Launch Analysis** from the **Actions** dropdown list in the **Details** pane.

3. Search by any unique identifier you used to label your samples during setup, select one or more samples to include in the analysis, then click **Next**.
4. *(Optional)* Select plugins to run with your analysis.

   Note: Oncomine Variant Annotator v2.4 is applied be default and cannot be edited or removed from the workflow.

   a. Click ⚙ adjacent to the selected plugin.

   b. Configure the plugin parameters as necessary, click **Submit**, then click **Close**.
5. Click **Next**.
6. Enter an **Analysis Name** and **Description** *(Optional)*, then click **Launch Analysis**.

The Ion Reporter™ Software performs the analysis and the results will appear on the **Analyses** page.

Customize the Ion Reporter™ workflow

The Ion Reporter™ workflow **Oncomine™ Tumor Mutation Load - w2.0 - DNA - Single Sample** calls variants at ≥5% allelic frequency at positions with read coverage ≥60 for Mutation Load calculation. Poor sample fixation can artificially increase the determined Mutation Load due to deamination. Deamination is reported as the Estimated SNP proportion consistent with Deamination (mainly FFPE) under the **Sample QC** tab. When considering the Estimated SNP proportion consistent with Deamination (mainly FFPE) number, note that samples with low tumor content may have true biological somatic C:G>T:A mutations that are counted towards deamination.

Samples of low quality that exhibit deamination can be analyzed using an analysis workflow with higher minimum allele frequency (for example, 10%) for Mutation Load calculation to reduce the impact of deamination on the reported Mutation Load. It is important to consider tumor content of a given sample when increasing the minimum allele frequency of the Mutation Load calculation. Samples with low tumor content can have many true somatic mutations that are excluded from the Mutation Load calculation when analyzed using a workflow with a higher minimum allele frequency filter. For samples with a high Estimated SNP proportion consistent with Deamination (mainly FFPE) number (for example, >100), Mutation Load values may not be reliable and adjusting the minimum allele frequency in the analysis parameters will not help. The mean depth and uniformity of the sample as determined by the coverageAnalysis plugin (see page 29) can also indicate sample quality.

To modify the existing Oncomine™ workflow to achieve higher minimum allele frequency for Mutation Load calculation, you must create a new filter chain, then copy and edit the Oncomine™ Tumor Mutation Load - w2.0 - DNA - Single Sample workflow as described.

1. In the **Workflows** tab, click **Presets**, then click **Create Presets** ▸ **Filter Chain**.
2. In the **Create Filter Chain** dialog, enter a **Name**, and **Description** *(not required)*.

   Note: The **Name** field accepts alphanumeric characters, underscore, hyphen, period, and space only.

3. Set the filters to apply to the filter chain.
   a. Click **Choose Filter**, type the filter name into the **Find Filter** field, then select the filter.
   b. Configure the filter with the provided selections.
   c. Click **Set**.
4. Repeat step 3 for each filter listed in the following table.

Filter name	Value	Selections	
		Include boundary values	Include unannotated variants
Filtered Coverage	GREATER_THAN = 60	Select	Select
Variant Type	SNV, INDEL	n/a	n/a
Minor Allele Frequency	From = 0.0, To = 0.000001	Select	Select
5000Exomes Global MAF (20161108)	From = 0.0, To = 0.000001	Select	Select

(continued)

Filter name	Value	Selections	
		Include boundary values	Include unannotated variants
ExAC GAF (1)	From = 0.0, To = 0.000001	Select	Select
UCSC Common SNPs	Filter value = Not In	n/a	n/a
Homopolymer Length	From = 0, To = 4	Select	Select
Allele Frequency	From = X[1], To = 1.0	Select	Select

[1] Set "From =" value to 0.05 for ≥5%, to 0.1 for ≥10% allele frequency

The **FilterChain Query** and **Selected Filters** panes should look like this.

FilterChain Query

Filtered Coverage AND Variant Type AND Minor Allele Frequency AND 5000Exomes Global MAF(20161108) AND ExAC GAF(1) AND UCSC Common SNPs AND Homopolymer Length AND Allele Frequency

Selected Filters

Name	Value
Filtered Coverage	Filtered Coverage >= 60
Variant Type	Variant Type in SNV, INDEL
Minor Allele Frequency	0.0 <= Minor Allele Frequency <= 0.000001
5000Exomes Global MAF(20161108)	0.0 <= 5000Exomes Global MAF(20161108) <= 0.000001
ExAC GAF(1)	0.0 <= ExAC GAF(1) <= 0.000001
UCSC Common SNPs	UCSC Common SNPs = Not In
Homopolymer Length	0 <= Homopolymer Length <= 4
Allele Frequency	0.1 <= Allele Frequency <= 1.0

5. Click **Save**, the **Filter Chain** is now available to apply to a custom workflow.

6. Copy, then edit the Oncomine™ Tumor Mutation Load - w2.0 - DNA - Single Sample workflow.
   a. In the **Workflows** tab, click **Overview**, then select the Oncomine™ Tumor Mutation Load - w2.0 - DNA - Single Sample workflow.
   b. Click **(Actions)** ▸ **Copy**.
   c. Click **Next** multiple times to advance to the **Parameters** step.

d. In the **Mutation Load Calculation Filter Chain** parameter dropdown list, select your new **Filter Chain**.

e. Click **Next**, then in the **Confirm** tab, enter a **Workflow Name** and description, then click **Save Workflow**.

The modified workflow is now available for use in the **Workflows** tab, **Overview** screen.

Remove deaminated bases from FFPE DNA

IMPORTANT! We have demonstrated that deaminated cytosine (uracil) bases can be enzymatically removed by treatment with Uracil-DNA Glycosylase (UDG) before the target amplification reaction of the Ion AmpliSeq™ Library Kit Plus library preparation protocol (page 15). However, the following protocol has not been fully verified for use with the Oncomine™ Tumor Mutation Load Assay. In a subset of Formalin-fixed paraffin-embedded (FFPE) samples tested, UDG treatment was demonstrated to reduce the Oncomine™ Tumor Mutation Load Assay Estimated SNP proportion consistent with Deamination (mainly FFPE) number.

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 Estimated SNP proportion consistent with Deamination number (see "Sample QC tab" on page 39) when determining the Mutation Load result. FFPE samples that yield a high Estimated SNP proportion consistent with Deamination number may benefit from treatment with Uracil-DNA Glycosylase (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	≤6.5 µL
UDG, heat-labile	1 µL
Low TE	to 7.5 µL

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

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

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

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

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

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

5. Carefully remove the plate seal, then proceed immediately to "Prepare DNA target amplification reactions" on page 15, adding the target amplification reaction components to the well containing 7.5 µL of UDG treated FFPE DNA.

D Example reports

Example Analysis Results Report

Analysis Report
Example Analysis Report

Background

Analysis

IX9_B810142L-T_Lib92_chip31_v1_c11954_2018-08-22-15-09-13-965

Ion Reporter Version	Launched by	Launched on	Workflow
5.10	Ion User	August 22, 2018 03:13 PM	Oncomine Tumor Mutation Load - w2.0 - DNA - Single Sample r.0

Annotations	Reference
Oncomine Tumor Mutation Load Assay Annotations v1 r.0	Oncomine Tumor Mutation Load Hotspots v2.0, hg19, Oncomine Tumor Mutation Load Regions v2.0, Oncomine Tumor Mutation Load Mask v2.0

Samples

IX9_B810142L-T_Lib92_chip31_v1

Gender	Relationship	Chip Type	Sample Type
Unknown	Proband	540	DNA

Reported Variants

Classification	Locus	Genotype	Filter
Unclassified	chr1:115256528	TTG/TCG	PASS
Ref	**Observed Allele**	**Type**	**Genes**
TTG	ATG,CAG,CCG,CTT,GTG,TAA,TAG, TCG,TCT,TGG	SNV	NRAS
Location	**Length**	**Oncomine Variant Class**	**Oncomine Gene Class**
NRAS:exonic:NM_002524.4	1	Hotspot	Gain-of-function
Info	**Variant ID**	**% Frequency**	
HS	COSM585;COSM586;COSM30646;COSM33693;COSM53223;COSM583;COSM584;COSM582;COSM12725;COSM579	ATG=0.00, CAG=0.00, CCG=0.00, CTT=0.00, GTG=0.00, TAA=0.00, TAG=0.00, TCG=14.89, TCT=0.00, TGG=0.00	

Variant Details

Gene: NRAS -- Exon: 3 --- chr1:115256528 --- NM_002524.4 --- Classification: Unclassified

Sample	Genotype	Amino Acid
IX9_B810142L-T_Lib92_chip31_v1	c.182A>G	p.Gln61Arg

* protein change takes into account changes at multiple genomic loci in same codon

Source	Description
UserName	Notes

Comments

Sign-Off

Disclaimer

Oncomine™ Tumor Mutation Load Assay report

Mutation Load
Analysis Report

Mutation Load (Mutations/Mb): 69.74

Analysis

B810145_C_T_v7_c1789_2018-07-12-16-16-46-121

Ion Reporter Version	Launched by	Launched on	Workflow
5.10	Ion User	July 12, 2018 04:16 PM	Oncomine Tumor Mutation Load - w2.0 - DNA - Single Sample r.0

Annotations	Reference
Oncomine Tumor Mutation Load Assay Annotations v1 r.0	Oncomine Tumor Mutation Load Hotspots v2.0, hg19, Oncomine Tumor Mutation Load Regions v2.0, Oncomine Tumor Mutation Load Mask v2.0

Samples

B810145_C_T_v7

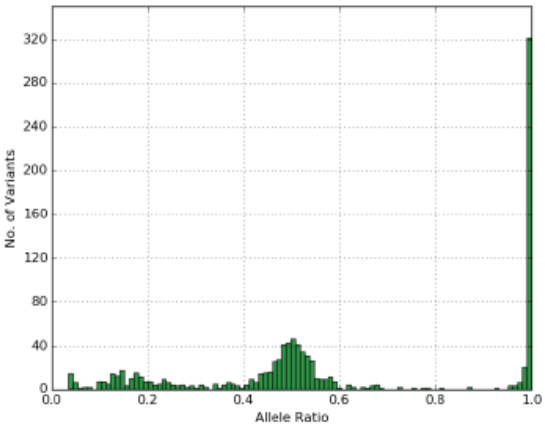
Gender	Relationship	Chip Type	Sample Type
Unknown	Proband	540	DNA

QC Metrics

Average Coverage	Total Variants Called	Estimated SNP proportion consistent with Deamination (mainly FFPE)
608.0	1,048	20

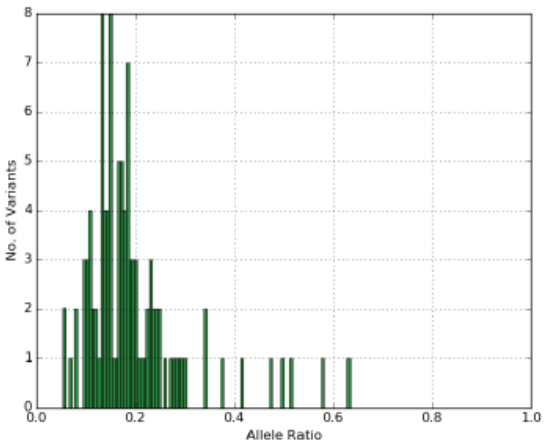
Analysis Results

Germ-line and Somatic Variants: 1,048



Only Somatic Variants: 108

Nonsynonymous: 59; Synonymous: 18



Number of Somatic Variants Present in COSMIC: 15

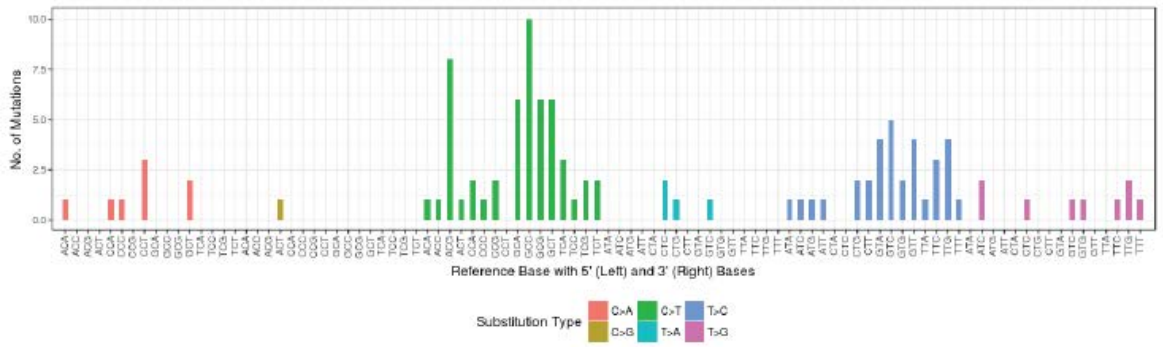
For Research Use Only. Not for use in diagnostic procedures.

Report generated by Ion User on Aug 02 2018 01:32 PM

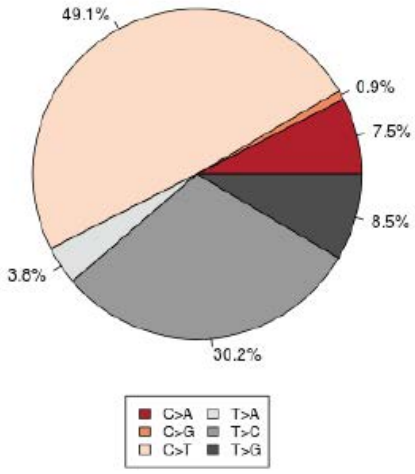
1 of 2

ThermoFisher SCIENTIFIC

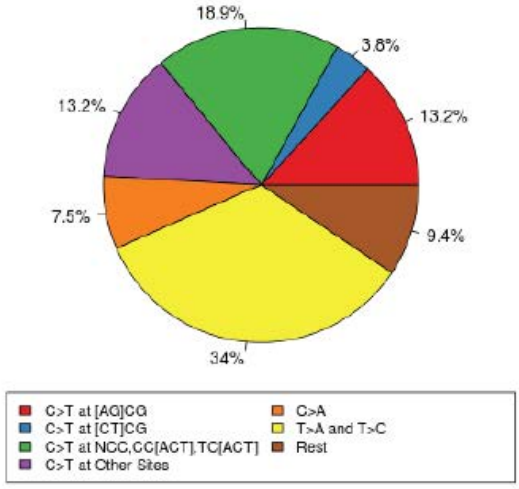
Substitution Type and Context of Somatic Mutations



Substitution Type of Somatic Mutations



Signature Pattern of Somatic Mutations



Additional Information:

High C>T at CpG is consistent with Spontaneous deamination of 5-methylcytosine[1]

High C>T at CpC, CpC, TpC, T>A, and T>C is consistent with UV damage[2]

High C>A is consistent with smoking damage[3]

High C>T (site independent) is consistent with FFPE processing[4]

[1] Alexandrov LB et al. *Nature*. 2013;[2]Hayward NK et al. Nature. 2017; [3]Alexandrov LB et al. *Cancer Etiology*. 2016;[4]Wong SQ et al. *BMC Medical Genomics*. 2014;

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 Kit Plus User Guide (Pub. No. MAN0017003)	Comprehensive instruction and troubleshooting for the preparation of Oncomine™ and Ion AmpliSeq™ libraries.
Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide (Pub. No. MAN0013432)	Describes the automated preparation and templating of Oncomine™ Tumor Mutation Load Assay libraries using the Ion Chef™ System.
Ion 540™ Kit – Chef User Guide (Pub. No. MAN0010851)	Describes the automated template preparation of Oncomine™ Tumor Mutation Load Assay libraries using the Ion Chef™ System for sequencing on an Ion GeneStudio™ S5 Series System or Ion S5™/Ion S5™ XL System.
Ion 550™ Kit – Chef User Guide (Pub. No. MAN0017275)	Describes the automated template preparation of Oncomine™ Tumor Mutation Load Assay libraries using the Ion Chef™ System for sequencing on an Ion GeneStudio™ S5 Series System or Ion S5™ XL System. The Ion 550™ Chip is not compatible with the Ion S5™ Sequencer.
Ion Reporter™ Software 5.10 User Guide (Pub. No. MAN0017605)	Includes software installation and set up information in addition to Ion Reporter™ Software Help content.
Torrent Suite™ Software 5.10 User Guide (Pub. No. MAN0017598)	Includes software installation and set up information in addition to Torrent Suite™ Software Help content.
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.

Note: For additional documentation, see “Customer and technical support” on page 64.

Customer and technical support

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

- Worldwide contact telephone numbers
- Product support information
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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

References

Alexandrov, LB et al. (2013) Signatures of mutational processes in human cancer. *Nature* 500:415–421.

Alexandrov, LB et al. (2016) Mutational signatures associated with tobacco smoking in human cancer. *Science* 354:618–622.

Hayward, NK et al. (2017) Whole-Genome Landscapes of Major Melanoma Subtypes. *Nature* 545:175–180.

Wong, SQ et al. (2014) Sequence artefacts in a prospective series of formalin-fixed tumours tested for mutations in hotspot regions by massively parallel sequencing. *BMC Medical Genomics* 7:23.