ion torrent

Oncomine™ Focus Assay

USER GUIDE

for use with the Oncomine™ Focus Assay, AmpliSeq™ Library or Oncomine™ Focus Assay, Chef-Ready kits

Catalog Numbers A29230, A35957, A42008

Publication Number MAN0015819

Revision D.0

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

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

Revision history: MAN0015819 D.0 (English)

Revision	Date	Description
D.0	18 September 2023	Updated Ion AmpliSeq™ Kit for Chef DL8 (Cat. No. A29024) to include the PCR Plate Frame.
C.0	11 December 2018	• Three user guide set consolidated into a single user guide. • Include instruction for library preparation with the Ion Chef™ System. • Updated for use with Oncomine™ Reporter Software 4.1.
B.0	9 March 2018	Update to cDNA preparation protocol: • Oncomine™ Focus RNA Assay used at 0.5X final concentration when preparing libraries manually • updated thermal cycling parameters—decreased denaturation temperature to 98°C for cDNA amplification These changes to the protocol may improve the sequencing metrics and potentially increase the calling rate of fusions by reducing false negatives.
A.0	22 August 2016	*Oncomine™ Focus Assay User Guide*. Provides detailed, step-by-step instructions on how to create an Oncomine™ Focus Assay library.

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

Contents

1 Product information

Product description

The Oncomine™ Focus Assay is a targeted multi-biomarker next-generation sequencing (NGS) assay that enables the simultaneous detection of hundreds variants across 52 genes relevant to solid tumors. This assays allows concurrent analysis of DNA and RNA to simultaneously detect multiple types of variants, including hotspots, single nucleotide variants (SNVs), indels, copy number variants (CNVs) and gene fusions, in a single workflow. Designed for translational and clinical research, this assay includes solid tumor genes targeted by on-market oncology drugs and published evidence.

This guide covers library preparation from DNA or RNA using the Ion AmpliSeq™ Library Kit 2.0 and the Oncomine™ Focus DNA Assay and Oncomine™ Focus RNA Assay panels, which are included as part of the Oncomine™ Focus Assay. The assay can be used with barcoded adapters so that up to seven paired DNA and RNA samples plus DNA and RNA no template controls (NTCs) can be combined and loaded onto a single Ion Chip in a single workflow to minimize the per-sample sequencing cost.

This guide covers the following products:

- Oncomine™ Focus Assay, AmpliSeq™ Library (Cat. Nos. A35957, A29230)
- Ion Xpress™ Barcode Adapters (various Cat. Nos.)
- IonCode™ Barcode Adapters (Cat. No. A29751)
- Ion AmpliSeq™ Sample ID Panel (Cat. No. 4479790)
- SuperScript™ VILO™ cDNA Synthesis Kit (Cat. No. 11754050)

Note: The Oncomine™ Focus Assay, Chef-Ready library preparation kit (Cat. No. A42008) is also available for automated library preparation (see page 8). The kit provides the Oncomine™ Focus DNA Assay Panel (1-pool) and Oncomine™ Focus RNA Assay Panel (1-pool) at 2X concentration premeasured in barcoded Primer Pool tubes ready to load into an Ion AmpliSeq™ Kit for Chef DL8 cartridge.

Prerequisites

This guide also assumes that you have:

- A general understanding of Ion Torrent™ sequencing chemistry and workflow
- Knowledge of techniques for handling and preparing DNA libraries

Contents and storage

Oncomine™ Focus Assay, AmpliSeq™ Library

The 16-reaction Oncomine™ Focus Assay, AmpliSeq™ Library (Cat. No. A35957) consists of the 5X Oncomine™ Focus DNA Assay Panel and the 5X Oncomine™ Focus RNA Assay Panel, and 2 Ion AmpliSeq™ Library Kit 2.0 (Cat. No. 4475345) kits, for the rapid preparation of barcoded sample libraries from DNA and RNA, which you can then combine and sequence simultaneously.

The 48-reaction Oncomine™ Focus Assay, AmpliSeq™ Library (Cat. No. A29230) consists of the 5X Oncomine™ Focus DNA Assay Panel and the 5X Oncomine™ Focus RNA Assay Panel, and the Ion AmpliSeq™ Library Kit 2.0-96LV (Cat. No. A26120) kit, for the rapid preparation of barcoded sample libraries from DNA and RNA, which you can then combine and sequence simultaneously.

Contents	Amount		Storage
Oncomine™ Focus Assay, AmpliSeq™ Library	**Cat. No. A35957 (16 reactions)**	**Cat. No. A29230 (48 reactions)**	
5X Oncomine™ Focus DNA Assay	64 µL	192 µL	–30°C to –10°C
5X Oncomine™ Focus RNA Assay	64 µL	192 µL	
Ion AmpliSeq™ Library Kit 2.0	**Cat. No. 4475345 (8 libraries)**	**Cat. No. A26120 (96 libraries)**	
5X Ion AmpliSeq™ HiFi Mix (red cap)	40 µL	480 µL	–30°C to –10°C
FuPa Reagent (brown cap)	16 µL	192 µL	
Switch Solution (yellow cap)	32 µL	384 µL	
DNA Ligase (blue cap)	16 µL	192 µL	
Ion AmpliSeq™ Adapters (green cap)	16 µL	192 µL	
Platinum™ PCR SuperMix High Fidelity (black cap)	400 µL	3 × 1.6 mL	
Library Amplification Primer Mix (white cap)	16 µL	192 µL	
Low TE	6 mL	12 mL	15°C to 30°C[1]

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

Oncomine™ Focus Assay, Chef-Ready

The Oncomine™ Focus Assay, Chef-Ready Kit (Cat. No. A42008) provides the Oncomine™ Focus DNA Assay Panel and the Oncomine™ Focus RNA Assay Panel at 2X concentration pre-measured in barcoded Primer Pool tubes ready to load into an Ion AmpliSeq™ Chef Reagents DL8 cartridge. In addition, the kit provides all the reagents and supplies in an Ion AmpliSeq™ Kit for Chef DL8 (Cat. No. A29024) sufficient for preparing 32 libraries. For more information about preparing Oncomine™ Focus Assay libraries on the Ion Chef™ System, see the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432).

Component	Amount	Storage
Oncomine™ Focus DNA Assay, Chef-Ready (32 reactions)		
2X Oncomine™ Focus DNA Assay Panel	8 × 150 µL	–30°C to –10°C
Oncomine™ Focus RNA Assay, Chef-Ready (32 reactions)		
2X Oncomine™ Focus RNA Assay Panel	8 × 150 µL	–30°C to –10°C
Ion AmpliSeq™ Kit for Chef DL8 (Cat. No. A29024)		
Ion AmpliSeq™ Chef Reagents DL8	2 × 4 cartridges	–30°C to –10°C
Ion AmpliSeq™ Chef Solutions DL8	2 ×4 cartridges	2°C to 8°C[1]
Ion AmpliSeq™ Chef Supplies DL8 (per insert) • Ion AmpliSeq™ Tip Cartridge L8 • PCR Frame Seal • PCR Plate Frame • Enrichment Cartridge	2 boxes with 4 inserts	15°C to 30°C
IonCode™ 0101–0132 in 96 Well PCR Plates (dried) Set includes 4 PCR plates: • IonCode™ 0101–0108 in 96 Well PCR Plate (red) • IonCode™ 0109–0116 in 96 Well PCR Plate (yellow) • IonCode™ 0117–0124 in 96 Well PCR Plate (green) • IonCode™ 0125–0132 in 96 Well PCR Plate (blue)	2 sets of 4 plates	15°C to 30°C

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

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 1–16 Kit	A29751 4471250, 4474009, 4474518, 4474519, 4474520, 4474521, or 4474517
Agencourt™ AMPure™ XP Kit	Beckman Coulter, A63880 or A63881
(RNA only) SuperScript™ VILO™ cDNA Synthesis Kit	11754050
One of the following: • GeneAmp™ PCR System 9700 or Dual 96-well Thermal Cycler • Applied Biosystems™ 2720 Thermal Cycler • 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
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
Qubit™ 4 Fluorometer[1]	Q33238
96-well plate centrifuge	MLS
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	
TaqMan™ RNase P Detection Reagents Kit	4316831
Qubit™ dsDNA HS Assay Kit (DNA)	Q32851/ Q32854
Qubit™ RNA HS Assay Kit (RNA)	Q32855
Recommended for library quantification	
Ion Library TaqMan™ Quantitation Kit	4468802
Recommended controls	
Ion AmpliSeq™ Sample ID Panel	4479790
Horizon QMRS	HD200
AcroMetrix™ Oncology Hotspot Control	969056
Biochain CancerSeq™ Plus	T2235152-SC lot # B906046

[1] Qubit™ 2.0 & 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

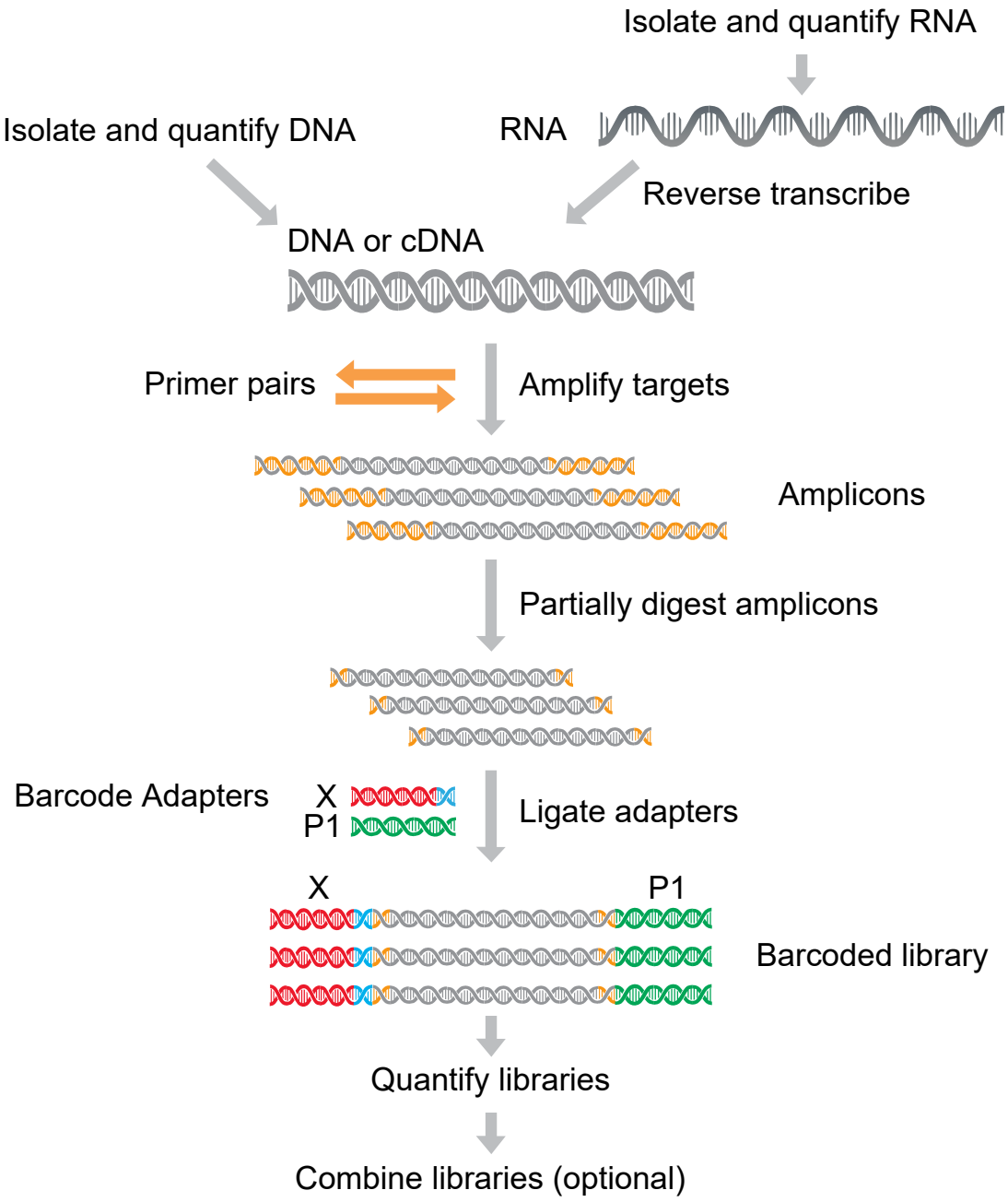
Procedural guidelines

- Minimize freeze-thaw cycles of Oncomine™ Comprehensive Assay Plus 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™ Side Magnet.

Before you begin

- Thaw components that contain enzymes—such as 5X Ion AmpliSeq™ HiFi Mix, FuPa Reagent, DNA Ligase, 10X SuperScript™ Enzyme Mix, and Platinum™ PCR SuperMix HiFi—on ice, and keep on ice during procedure. All other components, including primer pools, may be thawed at room temperature. Gently vortex and spin down before use.
- If there is visible precipitate in the 5X VILO™ Reaction Mix or Switch Solution after thawing, vortex or pipet up and down at room temperature to resuspend.

Library preparation from genomic DNA or RNA



3 Manual library preparation

Guidelines for RNA isolation and quantification

- Use kits in "Recommended materials" for isolating total RNA.
- We recommend the Qubit™ RNA HS Assay Kit (Cat. No. Q32855) for quantifying RNA.
- Each reverse transcription reaction requires 10 ng of DNase-treated total RNA (≥1.43 ng/µL).
- In general, library yield from high quality RNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.
- Increasing the amount of RNA will usually result in higher quality libraries, especially when RNA quality or quantity is unknown. We recommend using 1 ng total RNA only with high-quality, well-quantified samples.

Guidelines for DNA isolation and quantification

- See "Recommended materials" on page 10 for isolating gDNA.
- We recommend the TaqMan™ RNase P Detection Reagents Kit (Cat. No. 4316831) or Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) for quantifying amplifiable human genomic DNA.
- Spectrophotometric quantification methods 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, and low library yields.

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 using 1 ng gDNA (300 copies) only with high-quality, well-quantified samples.

RNA: Reverse transcribe and set up target amplification

Reverse transcribe RNA

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

Note: We recommend that PCR setup be done on ice or a cold block.

IMPORTANT! If there is visible precipitate in the 5X VILO™ Reaction Mix, vortex or pipet up and down to resuspend. Ensure that there is no precipitate in the tube cap.

1. For each sample, add the following components into a single well of a 96-well PCR plate. Prepare a master mix without sample RNA for multiple reactions.

Component	Volume
5X VILO™ Reaction Mix	2 µL
10X SuperScript™ Enzyme Mix	1 µL
Total RNA (10 ng)[1]	≤ 7 µL
Nuclease-free Water	to 10 µL
Total volume per well	**10 µL**

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

2. Seal the plate with a MicroAmp™ Clear Adhesive Film, vortex thoroughly, then centrifuge at 100 rcf for 30 seconds to collect droplets.

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

Temperature	Time
42°C	30 minutes
85°C	5 minutes
10°C	Hold

STOPPING POINT Samples can be stored at 10°C overnight (12–16 hours) in the thermal cycler. For longer periods, store at –20°C.

4. Centrifuge the plate at 100 rcf for 30 seconds before proceeding to the next step.

Prepare cDNA target amplification reactions

IMPORTANT! Primer pools and HiFi Mix are viscous. Pipet slowly and mix thoroughly.

1. Place the 96-well plate in a pre-chilled cold block or on ice.
2. Thaw the 5X Ion AmpliSeq™ HiFi Mix on ice, gently vortex to mix, then centrifuge briefly to collect.
3. Remove the seal from the plate, then add the following components to each cDNA synthesis reaction. Prepare a master mix for multiple reactions.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
5X Oncomine™ Focus RNA Assay[1]	2 µL
Nuclease-free Water	4 µL
Total volume per well (includes 10 µL from cDNA synthesis)	**~20 µL**

[1] Updated manual library preparation cDNA amplification protocol uses the 5X Oncomine™ Focus RNA Assay primers at 0.5X final concentration. Chef-ready library preparation does not require additional primer dilution.

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

Amplify the cDNA targets

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 enzyme	98°C	2 min
Cycle; set number according to the following table	Denature	98°C	15 sec
	Anneal and extend	60°C	4 min
Hold	—	10°C	Hold

Input nucleic acid[1]	Recommended cycle number (10 ng)	Cycle number adjustment[2]	
		1 ng RNA input	100 ng RNA input
High quality RNA	27	+3	–3
FFPE RNA	30	+3	–3

[1] Cycle numbers can be increased when input material quality or quantity is questionable.
[2] The recommended cycle number is based on 10 ng RNA input. Adjust the cycle number for lower or higher RNA input.

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.

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

Set up DNA target amplification reactions

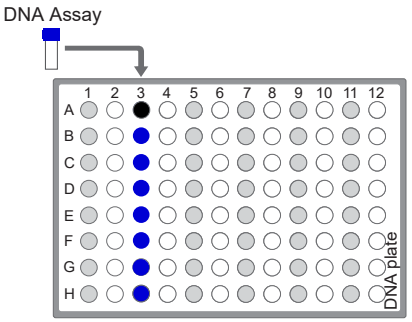
Prepare DNA target amplification reactions

IMPORTANT! Primer pools and HiFi Mix are viscous. Pipet slowly and mix thoroughly.

1. Place the 96-well plate in a pre-chilled cold block or on ice.
2. Thaw the 5X Ion AmpliSeq™ HiFi Mix on ice, gently vortex to mix, then centrifuge briefly to collect.
3. Add the following components to a single well of a 96-well PCR plate. Prepare a master mix without sample DNA for multiple reactions.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
5X Oncomine™ Focus DNA Assay	4 µL
DNA (10 ng)[1]	≤12 µL
(Optional) Ion AmpliSeq™ Sample ID Panel	1 µL
Nuclease-free Water	to 20 µL

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



- Sample DNA target amplification reaction
- No Template Control (NTC) target amplification reaction

4. Seal the plate with a new MicroAmp™ Adhesive Film.
5. 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 DNA targets

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 enzyme	99°C	2 min
Cycle; set number according to the following table	Denature	99°C	15 sec
	Anneal and extend	60°C	4 min
Hold	—	10°C	Hold

Input nucleic acid[1]	Recommended cycle number (10 ng)	Cycle number adjustment[2]	
		1 ng DNA input	100 ng DNA input
High quality gDNA	17	+3	–3
FFPE DNA	20	+3	–3

[1] Cycle numbers can be increased when input material quality or quantity is questionable.
[2] The recommended cycle number is based on 10 ng DNA input. Adjust the cycle number for lower or higher DNA input.

Note: Addition of the Ion AmpliSeq™ Sample ID Panel does not require adjustment to cycle number.

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.

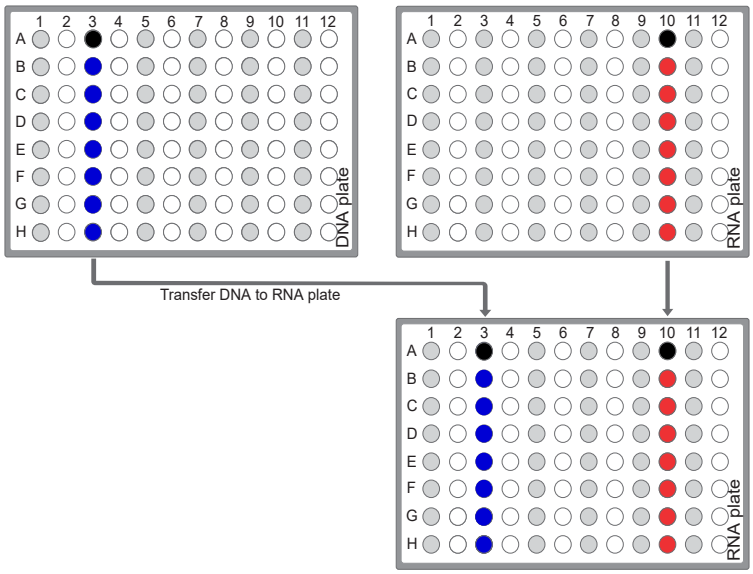
STOPPING POINT Target amplification reactions may be stored at 10°C overnight on the thermal cycler. For longer periods, store at –20°C.

Transfer the DNA amplicons

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

IMPORTANT! Be careful when removing the film to minimize contamination.

3. Transfer the amplicons from the DNA plate to the corresponding empty wells of the RNA/cDNA plate.



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

Partially digest amplicons

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

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

5. Briefly centrifuge the plate to collect the contents to the bottom of the wells.

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

Ligate adapters to the amplicons and purify

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

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

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

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

Ion Xpress™ Barcode Adapters only: Combine and dilute adapters

For each barcode X selected, prepare a mix of Ion P1 Adapter and Ion Xpress™ Barcode X at a final dilution of 1:4 for each adapter. Scale volumes as necessary. Use 2 µL of this barcode adapter mix in step 3 in "Perform the ligation reaction" on page 22.

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

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

[1] X = barcode chosen

Note: Store diluted adapters at –20°C.

Perform the ligation reaction

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

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

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

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

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

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

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

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

Purify the library

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

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

1. 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 and dilute the library". Alternatively, quantify the libraries using a Qubit™ Fluorometer, see Appendix C, "Quantify the amplified library with the Qubit™ Fluorometer".

Elute and dilute the library

1. Remove the plate with purified libraries from the plate magnet, then add 50 µL of Low TE to the pellet to disperse the beads.

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

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

4. Place the plate on the magnet for at least 2 minutes.

   STOPPING POINT Libraries can be stored at 4–8°C for up to 1 month. For longer term, store at –20°C. We recommend transferring the supernatant to a 1.5-mL Eppendorf LoBind™ tube for long-term storage.

5. Prepare a 100-fold dilution for quantification. Remove 2 µL of supernatant, containing the library, then combine with 198 µL of Nuclease-free Water.

Proceed immediately to "Quantify library by qPCR and calculate dilution factor" on page 25.

Quantify library by qPCR and calculate dilution factor

Determine the concentration of each Oncomine™ Focus Assay library by qPCR with the Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802). Analyze each sample, standard, and negative control in triplicate 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 and determination of the dilution factor that results in a concentration of ~50 pM, dilute the libraries to 50 pM.

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	Nuclease-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 triplicate 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	384-well plate
2X TaqMan™ Master Mix	10 µL	5 µL
20X Ion TaqMan™ Assay	1 µL	0.5 µL
Total	**11 µL**	**5.5 µL**

3. In an Optical PCR plate setup triplicate PCR reactions for each sample, standard, and NTC. To each well add the following components:

Component	Volume per reaction	
	96-well plate	384-well plate
PCR Master Mix	11 µL	5.5 µL
1:100 dilution of the sample[1]	9 µL	4.5 µL

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

4. Program your 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 OR 384-well Standard	Fast	Hold (UDG incubation)	50°C	2 min
		Hold (polymerase activation)	95°C	20 sec
		Cycle (40 cycles)	95°C	1 sec
			60°C	20 sec
96-well Standard	Standard	Hold (UDG incubation)	50°C	2 min
		Hold (polymerase activation)	95°C	2 min
		Cycle (40 cycles)	95°C	15 sec
			60°C	1 min

5. Following qPCR, calculate the average concentration of the undiluted Oncomine™ Focus Assay library by multiplying the determined concentration × 100.

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

   For example:

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

7. Dilute library to 50 pM as described, combine (see page 27), then proceed to template preparation, or store libraries as described below.

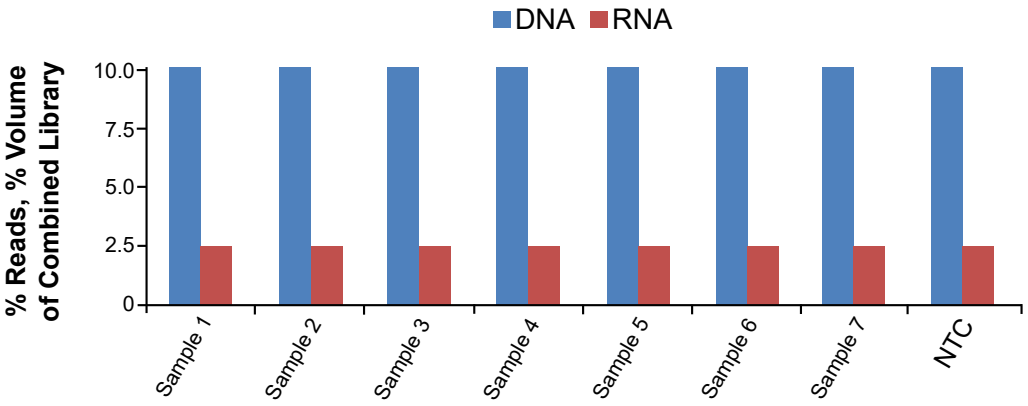
Note: A library that yields less than 50 pM can be rescued with library amplification. Combine 25 µL of unamplified library with 72 µL of Platinum™ PCR SuperMix HiFi and 3 µL of Library Amplification Primer Mix. Perform 5–10 library amplification cycles (see step 4 of "Amplify the library" on page 61 for cycling conditions).

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 DNA and RNA libraries to obtain different numbers of reads

When comparing libraries generated from genomic DNA and RNA, one million reads may be desired for the DNA sample, although only 250,000 reads may be preferred to evaluate RNA fusions in the same tissue sample. Unequal volumes of libraries can be combined to produce disproportionate numbers of reads. Use this strategy for combining DNA and RNA libraries that are prepared from the same sample to adjust the number of reads as desired. If the chip capacity is sufficient, multiple DNA/RNA sample-pair libraries can be combined in a single chip, as described in the following table.

Oncomine™ Focus Assay libraries with unique barcodes that are prepared from DNA and RNA from multiple samples should be diluted to 50 pM each, then combined at an 80:20 (DNA:RNA) ratio. The concentration of NTC libraries' is frequently less than 50 pM. For runs including an NTC library that is <50 pM, add in the same fractional volume of combined NTC library as is added of a 50-pM sample library. It is not necessary to reamplify an NTC library that is less than 50 pM.



Sample	Barcode	Fractional volume/ reads
DNA-1	BC_0101	0.1
RNA-1	BC_0102	0.025
DNA-2	BC_0103	0.1
RNA-2	BC_0104	0.025
DNA-3	BC_0105	0.1
RNA-3	BC_0106	0.025
DNA-4	BC_0107	0.1
RNA-4	BC_0108	0.025
DNA-5	BC_0109	0.1
RNA-5	BC_0110	0.025
DNA-6	BC_0111	0.1

(continued)

Sample	Barcode	Fractional volume/ reads
RNA-6	BC_0112	0.025
DNA-7	BC_0113	0.1
RNA-7	BC_0114	0.025
DNA-NTC	BC_0115	0.1
RNA-NTC	BC_0116	0.025
Sum	—	**1.0**

We recommend sequencing up to 7 samples plus 1 DNA NTC and 1 RNA NTC on a single Ion 520™ Chip, each sample consisting of one DNA library and one RNA library, that are combined at an 80:20 ratio (8 µL of DNA library + 2 µL of RNA library). A typical Oncomine™ Focus Assay sequencing run produces ~3.5 × 10^6 reads. If you need to combine libraries in a different way, you can determine the average number of reads per amplicon by doing a simple calculation. Example calculations are provided.

Example calculations

Example 1:

You have 7 samples, each consisting of one DNA library and one RNA library. We recommend the standard method of combining libraries at an 80:20 ratio (DNA:RNA). You can then calculate the average number of reads per amplicon as shown in the following table.

DNA	RNA
1. 3.5×10^6 reads/7 samples = 500,000 reads per combined library 2. 0.8[1] × 500,000 = 400,000 reads per DNA library 3. Average reads per amplicon: 400,000 reads per DNA library/269 amplicons[2] = 1487	1. 3.5×10^6 reads/7 samples = 500,000 reads per combined library 2. 0.2[3] × 500,000 = 100,000 reads per RNA library 3. Average reads per amplicon: 100,000 reads per RNA library/13 amplicons[4]= 7,692 **Note:** If a fusion is detected, the total number of amplicons will be 13 + N (N= the number of fusions detected).

[1] Proportion of the mixed library that is made up of DNA.
[2] Number of amplicons in the Oncomine™ Focus Assay.
[3] Proportion of the mixed library that is made up of RNA.
[4] Number of gene expression control amplicons in the Oncomine™ Focus Assay

Example 2:

You have 6 samples, including positive and non template controls (NTC), each consisting of one DNA library and one RNA library. We recommend the standard method of combining libraries at an 80:20 ratio (DNA:RNA by volume). You can then calculate the average number of reads per amplicon as shown in the following table.

This example assumes a negligible number of reads from the NTC sample libraries.

DNA	RNA
1. 3.5×10^6 reads/5 samples[1] = 700,000 reads per combined library 2. 0.8[2] × 700,000 = 560,000 reads per DNA library 3. Average reads per amplicon: 560,000 reads per DNA library/269 amplicons [3] = 2082	1. 3.5×10^6 reads/5 samples = 700,000 reads per combined library 2. 0.2[4] × 700,000 = 140,000 reads per RNA library 3. Average reads per amplicon: 140,000 reads per RNA library/13 amplicons[5]= 10,769 **Note:** If a fusion is detected, the total number of amplicons will be 13 + N (N= the number of fusions detected).

[1] 4 samples + 1 positive control sample
[2] Proportion of the mixed library that is made up of DNA.
[3] Number of amplicons in Oncomine™ Focus Assay.
[4] Proportion of the mixed library that is made up of RNA.
[5] Number of gene expression control amplicons in the Oncomine™ Focus Assay

Example 3:

You have 4 samples, each consisting of one DNA library and one RNA library, and an additional 4 RNA libraries that you want to re-sequence. We recommend combining all the DNA libraries together, and all the RNA libraries together, and finally combining the two at an 80:20 (DNA:RNA) ratio. You can then calculate the average number of reads per amplicon as shown in the following table.

DNA	RNA
1. 3.5×10^6 reads × 0.8 = 2.8×10^6 reads for all DNA libraries **2.** 2.8×10^6 reads/4 DNA libraries = 700,000 reads per DNA library **3.** Average reads per amplicon: 700,000 reads per DNA library/269 amplicons = 2602	**1.** 3.5×10^6 reads × 0.2 = 700,000 reads for all RNA libraries **2.** 700,000 reads/8 RNA libraries = 87,500 reads per RNA library **3.** Average reads per amplicon: 87,500 reads per RNA library/13 amplicons = 6,731 **Note:** If a fusion is detected, the total number of amplicons will be 13 + N (N= the number of fusions detected).
To combine the DNA and RNA libraries to yield similar average reads per amplicon, you will need to combine them at a different ratio: **Note:** This method can be preferable because it yields an equal or greater number of reads for each DNA and RNA library, preserving the sensitivity of the assay.	
1. Determine proportion of DNA amplicons (R_D) (R_D = # DNA amplicons/Total number of amplicons) $R_D = (269 \times 4)/((269 \times 4) + (13 \times 8)) = 0.91$ **2.** 3.5×10^6 reads × $R_D = 3.185 \times 10^6$ reads for 4 DNA libraries **3.** 3.185×10^6 reads/(4 libraries × 269 amplicons) = 2,960 average reads per amplicon	**1.** Determine proportion of RNA amplicons (R_R) (R_R = # RNA amplicons/Total number of amplicons) $R_R = (13 \times 8)/((269 \times 4) + (13 \times 8)) = 0.09$ **2.** 3.5×10^6 reads × $R_R = 3.15 \times 10^5$ reads for 8 RNA libraries **3.** 3.15×10^5 reads/(8 libraries × 13 amplicons) = 3,029 average reads per amplicon

Example 4:

You have only RNA samples and want to determine how many libraries you can combine in a single sequencing run and still maintain a good average number of reads per amplicon.

RNA
3.5×10^6 reads/100,000 reads per RNA library (calculated from example 1) = 35 RNA libraries **Note:** The limiting factor in combining libraries is the number of barcodes that are provided in each kit.

Example 5:

You have only DNA samples and want to determine how many libraries you can combine in a single sequencing run and still maintain a good average number of reads per amplicon.

DNA
3.5×10^6 reads/400,000 reads per DNA library (calculated from example 1) = 8.75 DNA libraries **Note:** We recommend that you combine no more than 7 DNA libraries for each sequencing run.

4 Chef Ready: Library preparation

Guidelines for RNA isolation and quantification

- Use kits in “Recommended materials” for isolating total RNA.
- We recommend the Qubit™ RNA HS Assay Kit (Cat. No. Q32855) for quantifying RNA.
- For library preparation with the Ion Chef™ System, each reverse transcription reaction requires 10 ng of DNase-treated total RNA (≥0.83 ng/µL).
- In general, library yield from high quality RNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.
- Increasing the amount of RNA will usually result in higher quality libraries, especially when RNA quality or quantity is unknown. We recommend using 1 ng total RNA only with high-quality, well-quantified samples.

Guidelines for DNA isolation and quantification

- See “Recommended materials” on page 10 for isolating gDNA.
- We recommend the TaqMan™ RNase P Detection Reagents Kit (Cat. No. 4316831) or Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) for quantifying amplifiable human genomic DNA.
- Spectrophotometric quantification methods 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, and low library yields.

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 using 1 ng gDNA (300 copies) only with high-quality, well-quantified samples.

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

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

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

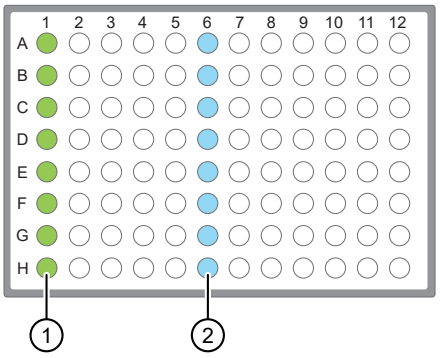
2. If the RNA was prepared from FFPE tissue and not previously heat-treated, heat at 80°C for 10 minutes, then cool to room temperature.

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

4. For each sample, add the following components into a single well in column 1 of the IonCode™ 96-well plate (provided in the Ion AmpliSeq™ Kit for Chef DL8). Prepare a master mix without sample RNA for multiple reactions.

Component	Volume
5X VILO™ Reaction Mix	2 µL
10X SuperScript™ Enzyme Mix	1 µL
Total RNA (10 ng)[1]	≤12 µL
Nuclease-free Water	to 15 µL
Total volume per well	**15 µL**

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



① Each column 1 well contains a 15 µL reverse transcription reaction, or no-template control reaction.

② Each column 6 well contains a dried-down IonCode™ Barcode Adapter. The lowest barcode number is in A6, and the highest is in H6. All appear light blue in the actual plates.

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

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

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

Temperature	Time
42°C	30 minutes
85°C	5 minutes
10°C	Hold

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

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

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

See “Ion Chef™ Instrument setup information for Chef Ready kit users” for information required to set up the Ion Chef™ Instrument.

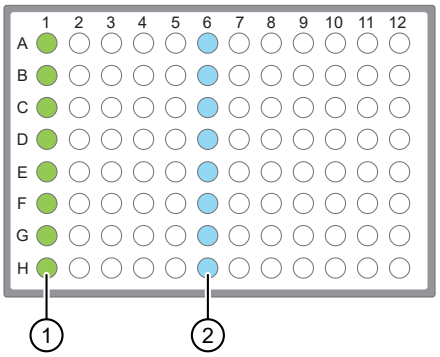
Add DNA to an IonCode™ PCR plate

1. Remove and discard the plate seal from an IonCode™ 96 Well PCR Plate.

2. For each sample, add the following components into a single well in column 1 of the IonCode™ 96-well plate (provided in the Ion AmpliSeq™ Kit for Chef DL8).

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

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



① Each column 1 well contains 15 µL of diluted gDNA sample (0.67 ng/µL, 10 ng total), or Nuclease-free Water as non-template control.

② Each column 6 well contains a dried-down IonCode™ barcode. The lowest barcode number is in A6, and the highest is in H6. All appear light blue in the actual plates.

Note:

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

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

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

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

For information on how to set up the Ion Chef™ Instrument, see “Ion Chef™ Instrument setup information for Chef Ready kit users”.

Ion Chef™ Instrument setup information for Chef Ready kit users

During Ion Chef™ Instrument setup, enter the following parameters when prompted. DNA samples will require between 18–21 cycles of target amplification and RNA samples will require between 28–31 cycles of target amplification. Samples of lower quality may require use of cycling parameters near the high end of the range.

Stating material	# of primer pools	Target amplification cycles	Anneal & extension time
DNA[1]	1	18	4 minutes
FFPE DNA[1]	1	21	4 minutes
RNA (cDNA)[1]	1	28	4 minutes
FFPE RNA[1]	1	31	4 minutes

[1] If both high quality and low quality nucleic acids are being used in the same reaction, use the low quality parameters.

Note: Chef-ready primers are provided at 2X concentration and must not be diluted further prior to loading on the Ion Chef™ Instrument.

5 Create a Planned Run

IMPORTANT! This kit is compatible with Torrent Suite™ Software 5.6 or later and Ion Reporter™ Software 5.2 or later. Before proceeding, we recommend that you update to the latest available versions of Torrent Suite™ Software, Ion Reporter™ Software, and Ion Chef™ System software. Contact your service representative for help with upgrading the software.

Note: The IonReporterUploader 5.2 plugin must be installed in Torrent Suite™ Software. If your Ion Reporter™ Software account is not configured, configure it through the software settings (⚙▾) see "Configure the IonReporterUploader plugin in Torrent Suite™ Software" on page 58).

About planned runs

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

In Torrent Suite™ Software 5.6, the primary Planned Run template to be used with the Oncomine™ Focus Assay is the **Oncomine™ Focus DNA and Fusions for S5** template. The **Oncomine™ Focus DNA for S5** and **Oncomine™ Focus Fusions for S5** templates can be used when you do not have sample pairs.

Application	Torrent Suite™ Software template	Description
DNA and Fusions	Oncomine™ Focus DNA and Fusions for S5	DNA and RNA planned run template
	Oncomine™ Focus Fusions for S5	RNA-only planned run template
Ion AmpliSeq™ DNA	Oncomine™ Focus DNA for S5	DNA-only planned run template

Create a custom Planned Run template

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.

The following instructions describe how to create a planned run template in which the DNA and RNA were prepared from the same sample. For more information on creating planned runs with other sample configurations 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 **DNA and Fusions** in the left navigation menu.
3. In the **DNA and Fusions** list, find the **Oncomine™ Focus DNA and Fusions for S5** Planned Run template from which you want to create your custom Planned Run, then click **(Actions)** ▸ **Copy**. The **Copy Template** workflow opens to the **Save** step.
4. Enter or select the required information in each field in the **Save** step.

Field	Action…
Template Name	Enter a name for the Planned Run template.
DNA Reference Library	Select **hg19(Human (hg19))**.
Set as Favorite	Select the **Set as Favorite** checkbox to add your custom template to the **Favorites** list.
DNA Target Regions[1]	Select **Oncomine_Focus.20160219.designed.bed**
DNA Hotspot Regions[2]	—
Fusions Reference Library[1]	Select **Oncomine_Focus_designs_072117_Reference(Oncomine Focus RNA Reference 072117)**
Fusions Target Regions[1]	Select **Oncomine_Focus_designs_072117_Reference.bed**

[1] Check with your service representative for updates to ensure the most current files are being used. For BED file installation instructions, see page 59.

[2] Not required for the Oncomine™ Focus Assay. Leave this field blank if a DNA Hotspot Regions file is not used.

5. Click the **Ion Reporter** step, then select your Ion Reporter™ account.

Note: If the Ion Reporter™ account is not configured, configure it through Ion Reporter Configure settings (see "Configure the IonReporterUploader plugin in Torrent Suite™ Software" on page 58 for more information).

6. In the **Existing Workflow** dropdown list, select **Oncomine™ Focus - 520 - w2.4 - DNA and Fusions - Single Sample** , then click **Next**.

Note: The Ion Reporter™ Software will not auto-analyze the data if the user does not select a workflow.

7. In the **Research Application** step, verify that **DNA and Fusions (Separate Libraries)** and **AmpliSeq DNA and Fusions** are selected for **Application** and **Target Technique** respectively, then click **Next**.

8. In the **Kits** step, verify the **Ion Chef Template Kit** radio button is selected, and the following fields are completed:

Field	Selection	
	Manual library preparation	Chef-ready library preparation
Instrument	Ion GeneStudio™ S5 System	
Library Kit Type	Ion AmpliSeq™ 2.0 Library Kit	Ion AmpliSeq™ Kit for Chef DL8
Template Kit	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef	
Sequencing Kit	Ion S5™ Sequencing Kit	
Base Calibration Mode	Default Calibration	
Chip Type	Ion 520™ Chip	
Barcode Set	Ion Xpress™	IonCode™ Barcodes 1–32
Flows	400	

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

10. Review the **Plugins** and **Projects** steps and make selections appropriate to your run, then click **Next**.

11. In the **Save** step, click **Copy Template** to save the new run template.

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

Create a Planned Run from a template

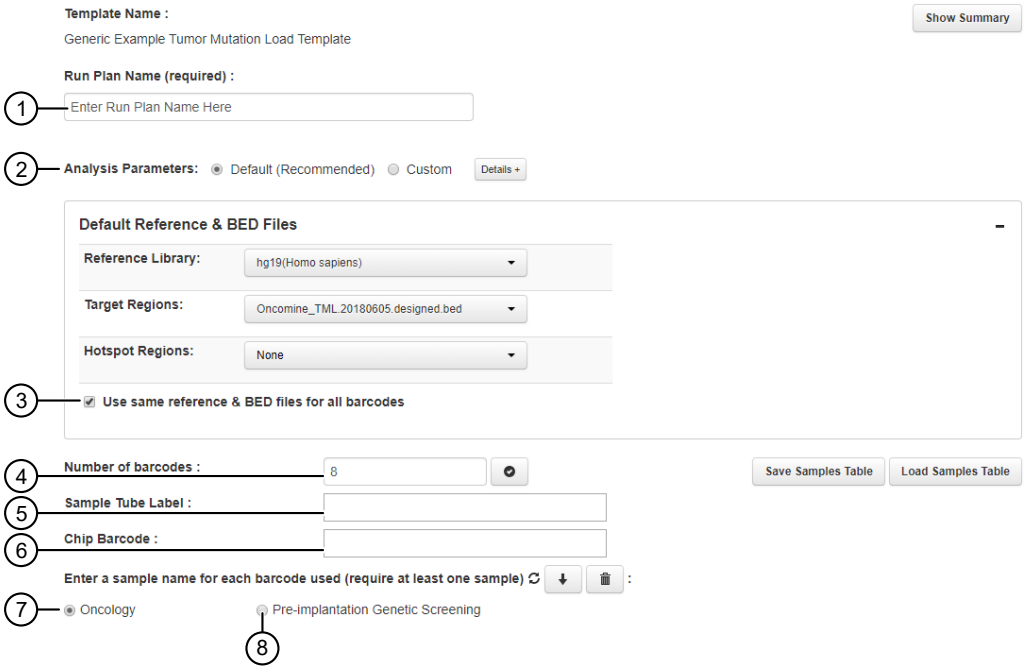
1. Sign in to the Torrent Suite™ Software.

2. In the **Plan** tab, click **Templates**, then click **DNA and Fusions** in the left navigation menu.

3. In the **DNA and Fusions** 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	Same sample for DNA and Fusions?	Ensure that the checkbox is selected.
5	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.
6	Sample Tube Label	Enter or scan the barcode of the Ion Chef™ sample tube that will be used in the run.
7	Chip Barcode	No entry required.
8	Oncology	Ensure that the radio button is selected.
9	Pre-implantation Genetic Screening	Ensure that the radio button is unselected.

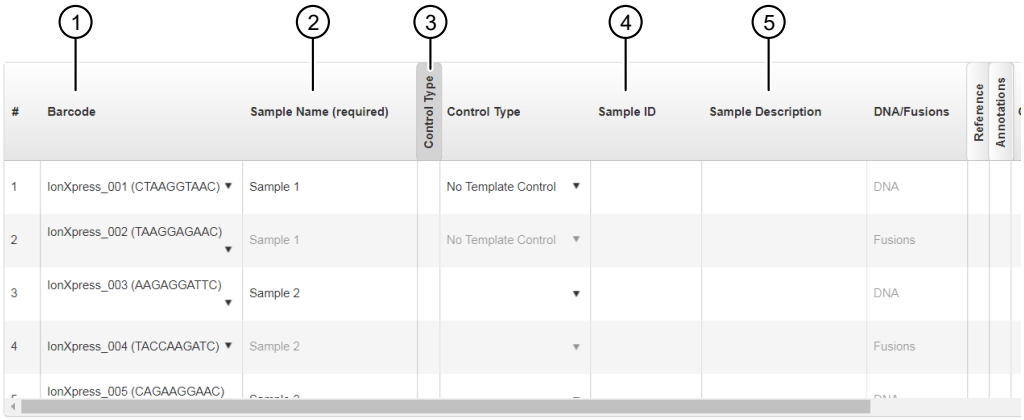


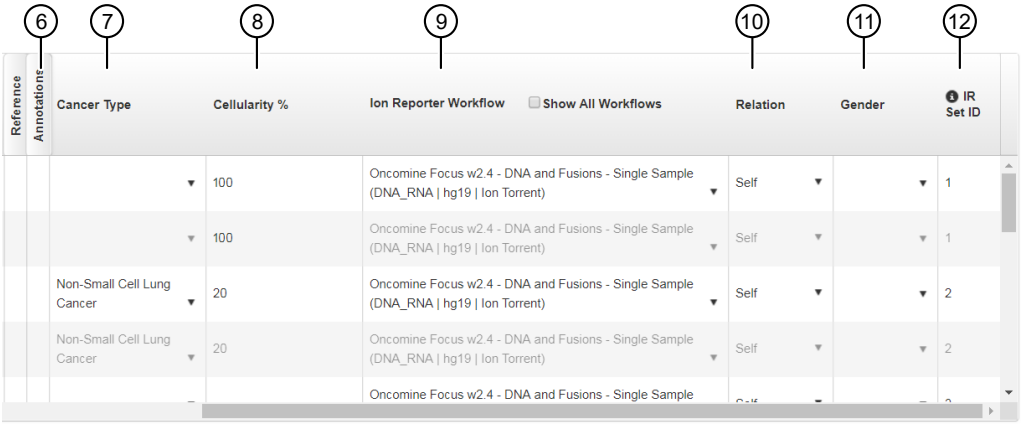
5. Enter sample information. Row numbers in the table correspond to the callouts in the following figure.

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. **Note:** The Oncomine™ Reporter Software will not auto-analyze the data if a Cancer Type is not selected. The Cancer Type can be added in the Oncomine™ Reporter Software. For more information, see "Create a report" on page 52.
8	Cellularity %	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.
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.

IMPORTANT! No template controls (NTCs) that are incorrectly designated will result in Ion Reporter™ Software analysis failure.





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.

6 Guidelines for templating and sequencing

Proceed to template preparation and sequencing using the kits and user guides indicated for your instrument setup.

Ion GeneStudio™ S5 Series Systems

Proceed to template preparation and sequencing using the following kits.

System	Kit	User Guide
Templating		
Ion Chef™ System	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef (Cat. Nos. A34019 or A34461[1])	*Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide* (Pub. No. MAN0016854)
Sequencing		
Ion GeneStudio™ S5 Series System	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef (Cat. Nos. A34019 or A34461[1])	*Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide* (Pub. No. MAN0016854)

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

To create a specific Run Plan for use in templating and sequencing see Chapter 5, “Create a Planned Run”. For more information, see the *Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide* (Pub. No. MAN0016854).

7 Variant analysis

IMPORTANT! If you are using the Ion Reporter™ Software version 5.2, you must have an off-cycle software package installed by your service representative to perform Oncomine™ Focus Assay Ion Reporter™ variant analysis. We recommend updating to the latest available version of Ion Reporter™ Software.

Analysis workflows in Ion Reporter™ Software

If your Planned Runs were set up correctly in Torrent Suite™ Software, automated analysis has already been performed and you can view the Oncomine™ analysis results in Ion Reporter™ Software. For instructions on manually launching an analysis, see "Manually launch an analysis" on page 44.

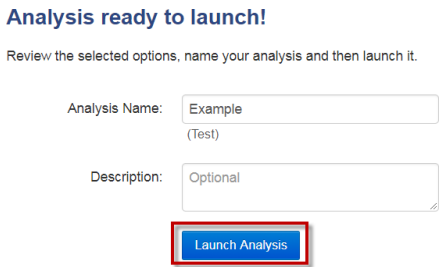
Note: Microsoft™ Excel™, or other spreadsheet tool, is required for viewing .vcf, .csv and .tsv files.

Available workflows in Ion Reporter™ Software include:

Analysis Workflow	Description
Ion Reporter™ Software 5.10 workflows	
Oncomine™ Focus - 520 - w2.4 - DNA and Fusions - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries, as well as gene fusions from targeted RNA libraries, of the Oncomine™ Focus Assay.
Oncomine™ Focus - 520 - w2.4 - DNA - Single Sample	Detects and annotates low frequency somatic variants (SNPs, InDels, CNVs) from targeted DNA libraries of the Oncomine™ Focus Assay.
Oncomine™ Focus - 520 - w2.4 - Fusions - Single Sample	Detects and annotates gene fusions from targeted RNA libraries of the Oncomine™ Focus Assay.
Oncomine™ Focus - 520 - w2.4 - Annotate Variants - Single Sample	Annotates VCF files from the Oncomine™ Focus Assay.

Manually launch an analysis

1. Sign in to the Ion Reporter™ Software.
2. In the **Workflows** tab, in the **Overview** screen, select **DNA and Fusions** from the **Research Application** dropdown list.
3. Type *Focus* in the search field, then click **Go** (or press Enter).
4. In the **Workflow Name** column, click the appropriate workflow (e.g., Oncomine™ Focus - 520 - w2.4 - DNA and Fusions - Single Sample), then click **Actions** ▸ **Launch Analysis** in the **Details** pane.
5. In the **Samples** step, search by any unique identifier you used to label your samples during setup, then ensure the sample's **Cellularity %** and **Sample Type** are defined.
6. Click the checkbox to select a DNA sample and a Fusions sample.
7. In the **Sample Groups** pane, click **Add Samples** to add the selected samples to a sample group.
8. Enter a **Group Name**, click **Add to Analysis**, then click **Next**.
9. In the **Plugins** step, ensure that the **Oncomine™ Variant Annotator v2.1 or later** plugin is selected, then click **Next**.
10. *(Optional)* Enter an **Analysis Name** and **Description**, then click **Launch Analysis**.

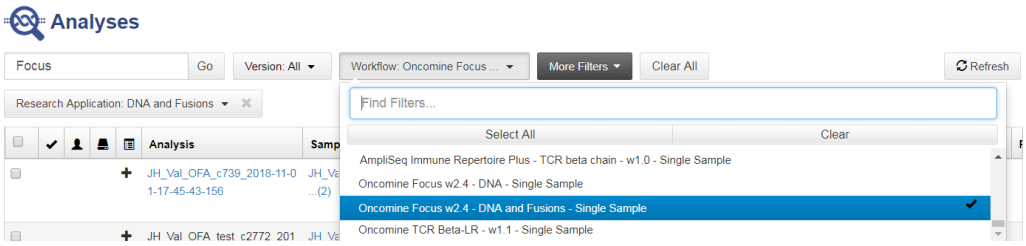


View results

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

1. Sign in to the Ion Reporter™ Software.
2. Click the **Analyses** tab.
   The **Overview** screen displays a list of analyses in the **Analyses** table.

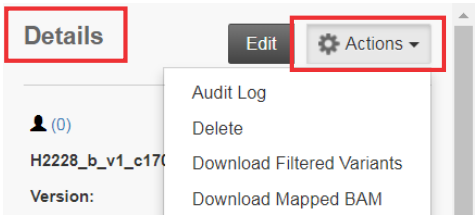
3. In the **Workflow** dropdown list, select **Oncomine™ Focus - 520 - w2.4 - DNA and Fusions - Single Sample** to limit the list of results to analyses using this workflow.



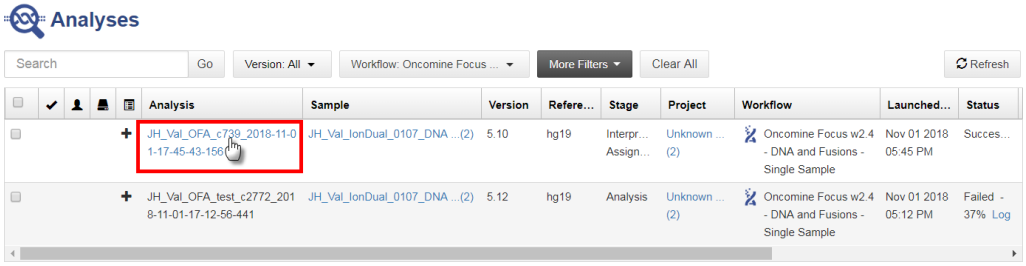
You can further refine the list of analyses by applying **More Filters**, or clicking column headers. The **Analyses** table automatically filters the content appropriate to your selection.

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

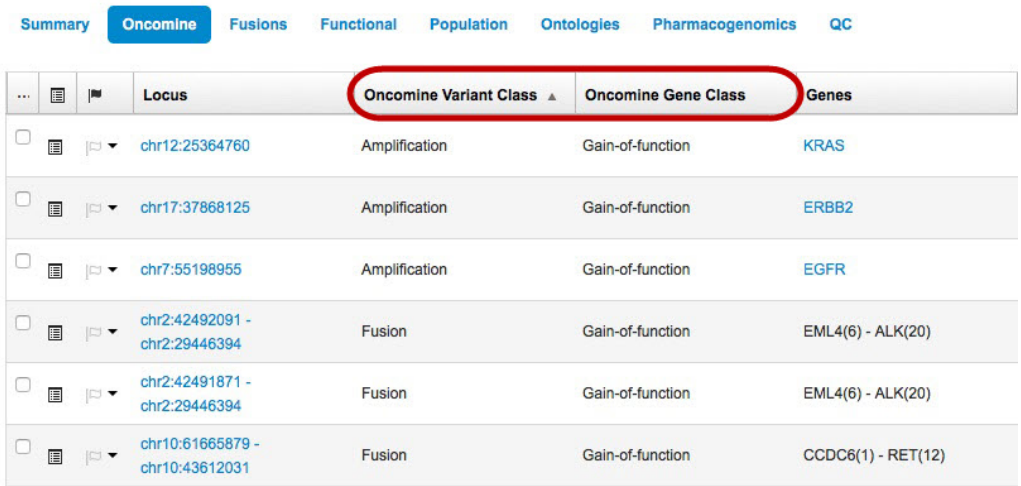
   In the **Details** pane you can view **Workflow Details**, and access the **Actions** dropdown list.



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



The **Analysis Results** page opens to the **Oncomine** tab displaying only Oncomine™ annotated variants that are known to be cancer drivers.

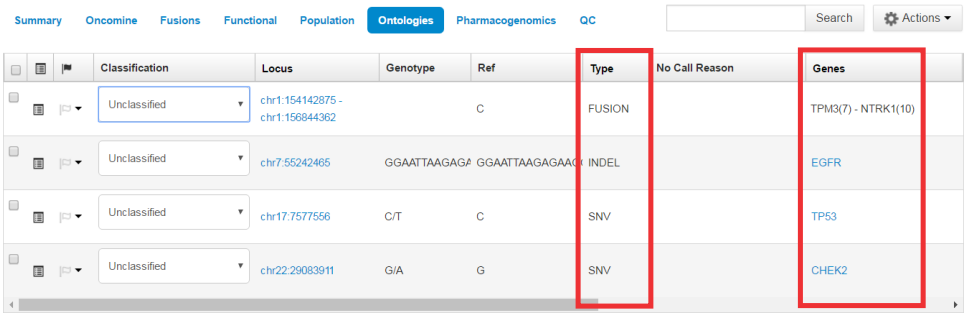


6. In the **Analysis Results** table, sort or filter the data using the Oncomine™-specific annotations. See the Ion Reporter™ Software help menu for more options.
   a. In the **Filter Options** pane, select the desired **Filter Chain**.

   Note:
   - The default **Filter Chain** is **Oncomine Variants, 5% CI CNV ploidy ≥ gain of 2 over normal (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 on filter chains, see the *Ion Reporter™ Software 5.10 User Guide* (Pub. No. MAN0017605).
   - Select **No Filter** to view all the variant calls attempted by the variant caller.
   - Saving the analysis using a filter chain other than **Oncomine Variants, 5% CI CNV ploidy ≥ gain of 2 over normal (5.10)** changes the variant calls that are saved in the VCF file and can affect downstream workflows, such as with Oncomine™ Reporter Software.

   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**.



After you have reviewed, filtered, and sorted your Analysis Result you can create a report (see "Generate a final report" on page 47), or download files for use by the Oncomine™ Reporter Software (see "Download Ion Reporter™ annotation VCF or TSV files" on page 47).

Generate a final report

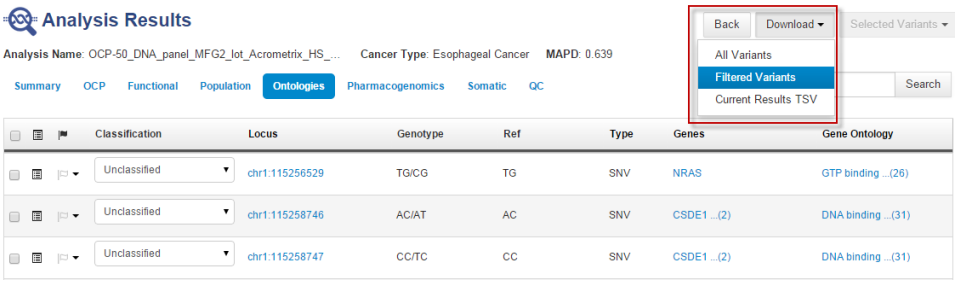
After you have reviewed, filtered, and sorted your analysis results, you can download a final report. The procedure described here includes creating and formatting a final report template.

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

Download Ion Reporter™ annotation VCF or TSV files

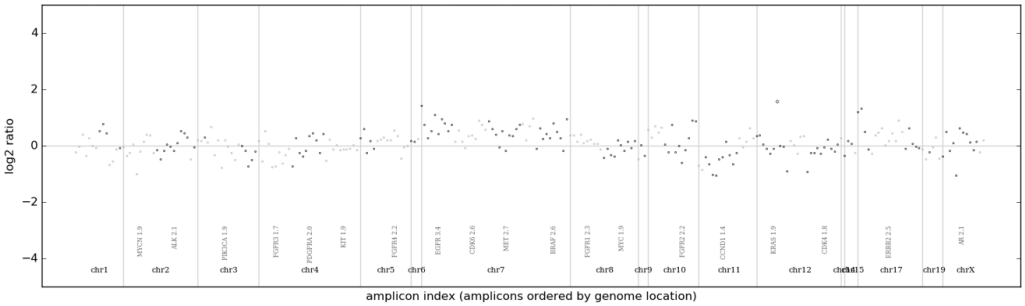
Variant call format (VCF), and tab separated values (TSV) files of the complete or filtered results can be downloaded from the **Analysis Results** screen.

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



2. Click **Home** ▸ **Notifications** to open the **Notifications** screen, then click ⬇ next to the file name to download your results.
   Alternatively, select one or more rows, then click **Download**.

The software generates a ZIP file with 4 folders: CNV_VCIB, QC, Variants, and Workflow_Settings. Within the Variants folder, you'll find the Oncomine™ annotated VCF file.

Folder	Contents of folder
CNV_VCIB	Contains an image file (`cn_results.png`) of the copy number determination for all amplicons. 
QC	Contains a PDF of the QC report, and a folder containing coverage statistics files.
Variants	Contains a folder with: • Intermediate and Oncomine™ annotated .VCF files, which are used by Oncomine™ Reporter Software. For more information, see the *Oncomine™ Reporter User Guide* (Pub. No. MAN0018068). • 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.

Oncomine™ Focus Assay annotations

The following annotations may be added in Ion Reporter™ software 5.2 or later.

Oncomine™ Gene Class	Oncomine™ Variant Class	Variant Type	Annotation Criteria
Gain-of-Function	Amplification	Copy Number Amplification	• Variant occurs in one of the 19 Oncomine™ Focus copy-gain genes • SVTYPE = "CNV" • Copy Number 5% CI fold change over normal is ≥2 (e.g., Copy Number 5% CI value is ≥4 when 2 copies are expected)
	Fusion	Gene Fusion	• Positive fusion call (SVTYPE = "Fusion" and FILTER = "PASS") in one the 271 Oncomine™ Focus fusion variants

(continued)

Oncomine™ Gene Class	Oncomine™ Variant Class	Variant Type	Annotation Criteria
Gain-of-Function	Hotspot	Gain of Function Missense Hotspot Mutation	• Variant's functional impact is missense • Variant occurs in one of 35 hotspot genes on the assay • Variant's transcript and codon position occur in pre-defined missense hotspot list • Allele Frequency (AF) ≥0.05, Alternate Allele Observation Count (FAO) ≥10
		Gain of Function In Frame Hotspot Mutation	• Variant occurs in one of 35 hotspot genes on the assay • Variant's function, transcript and coding syntax occur in pre-defined in-frame hotspot list • Allele Frequency (AF) ≥0.05, Alternate Allele Observation Count (FAO) ≥10
		Gain of Function Splice Site Hotspot Mutation	• Variant occurs in one of 35 hotspot genes on the assay • Variant's transcript, location, and exon occur in pre-defined splice site hotspot list • Allele Frequency (AF) ≥0.05, Alternate Allele Observation Count (FAO) ≥10

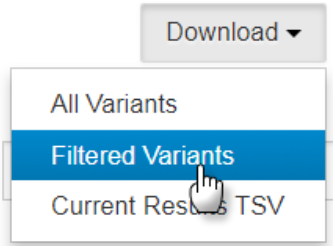
8 Generate an Oncomine™ Reporter report

For detailed instructions to create a report, see the *Oncomine™ Reporter User Guide* for software version 4.1 or later (Pub. No. MAN0018068).

Download the annotated VCF files from Ion Reporter™ Software

VCF (variant call format), or TSV (tab separated value) files of the complete or filtered results can be downloaded from the **Analysis Results** page. You can view the downloaded files individually, or upload a VCF file to a software application that accepts VCF files, such as Ion Torrent™ Oncomine™ Reporter software.

1. In the Ion Reporter™ Software, click the **Analyses** tab, then search, filter, or scroll to find your analysis in the **Analyses** table.
2. In the row of your analysis, click the link in the **Analysis** column.
   The **Analysis Results** table opens to the list of variants.
3. Click **Download** ▸ **Filtered Variants**.



4. In the **Home** tab, click **Notifications** to open the **Notifications** screen.
5. In the **Notifications** table, click **(Download)** in the **Download** column in the row of the appropriate notification to download your analysis results. To download multiple analysis results, select the checkbox in the row of one or more notifications, then click **Download** at the top right above the **Notifications** table.
   The software generates a ZIP file with 4 folders: CNV_VCIB, QC, Variants, and Workflow_Settings for each result. In the Variants folder, you will find the annotated VCF file.
6. Save the filtered Variants ZIP file to your local storage.

Upload Variant Call Format (VCF) files to Oncomine™ Reporter Software

The Oncomine™ Reporter Software enables access to a sample-specific variant report in three steps: **Upload**, **Filter**, and **Report**.

1. In the Oncomine™ Reporter Software home page, click **Upload**.
2. Click **Select File(s)**, then navigate to the previously downloaded Ion Reporter™ filtered variants ZIP file for the sample.

   Note: Selection of more than one file requires that they are saved to the same location. Alternatively, repeat step 2 and step 3 for each file required.

3. Select the ZIP file, then click **Open**.
4. *(Optional)* If you created an assay-specific Filter Preset. Select the **Filter Preset** you created for the Oncomine™ Focus Assay results from the dropdown list.

   Note: **Demo US & EU & Global Clinical Trials** is selected by default.

5. In the Assay dropdown list, select Oncomine™ Focus Assay.
6. Click **Upload & Review Content**.
7. *(If needed)* Select a **Cancer Type** from the dropdown list.

   Note: Assigning a cancer type in the Ion Reporter™ Software, or in the Torrent Suite™ Software Planned Run pre-populates this field. The data are auto-analyzed when you click **Upload & Review Content**.

   The **Content Review** screen opens with the list of biomarkers and relevant therapies that are identified in your sample. It combines information about driver variants from the VCF files with content from the Oncomine™ Knowledgebase.

Create a report

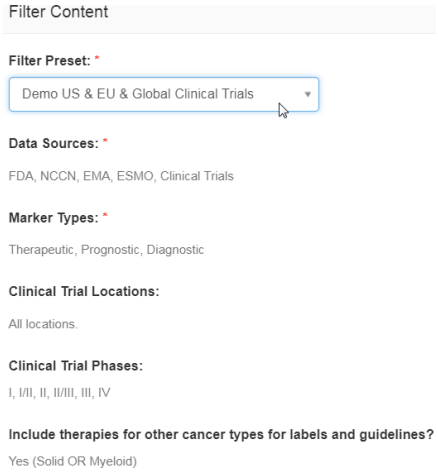
1. To change the cancer type, select from the **Cancer Type** dropdown list. This field is pre-populated if a cancer type was defined in the Ion Reporter™ Software, or selected in the Torrent Suite™ Software Planned Run.

2. Click **Filter** to view the details of the selected Filter Preset.

3. Select from the **Filter Preset** dropdown list to change the Filter Preset.

4. Review the information in the Tier Criteria Met, Therapeutic Guidelines, and Clinical Trials sections.
   a. Click a section to expand or collapse that section.

   b. Expand the **FDA-Approved Therapies** and/or **EMA-Approved Therapies** section. For approved labels, clicking the drug name link takes you to the label on the authority website.



   c. *(Optional)* Click the Trial ID to open **clinicaltrials.gov** with more information specific the individual clinical trial.

   d. *(Optional, configurator role only)* To exclude a clinical trial from the report, expand the **Clinical Trials** section, then deselect the checkbox next to the clinical trial. Alternatively, click **Unselect all** to remove all the trials from the report.

   e. *(Optional, configurator role only)* Edit a Filter to expand or reduce the evidence that is displayed in your report. Click **Filter** to reveal all the parameters. In the **Filter Content** section, edit the parameters, then click **Apply**. For more information, see the *Oncomine™ Reporter User Guide* for version 4.1 or later of the software (Pub. No. MAN0018068).

5. Click **Generate Report**, then select from the report settings to configure your report.
   a. Select a **Report Template** from the dropdown list.

   b. Enter a filename for the report.

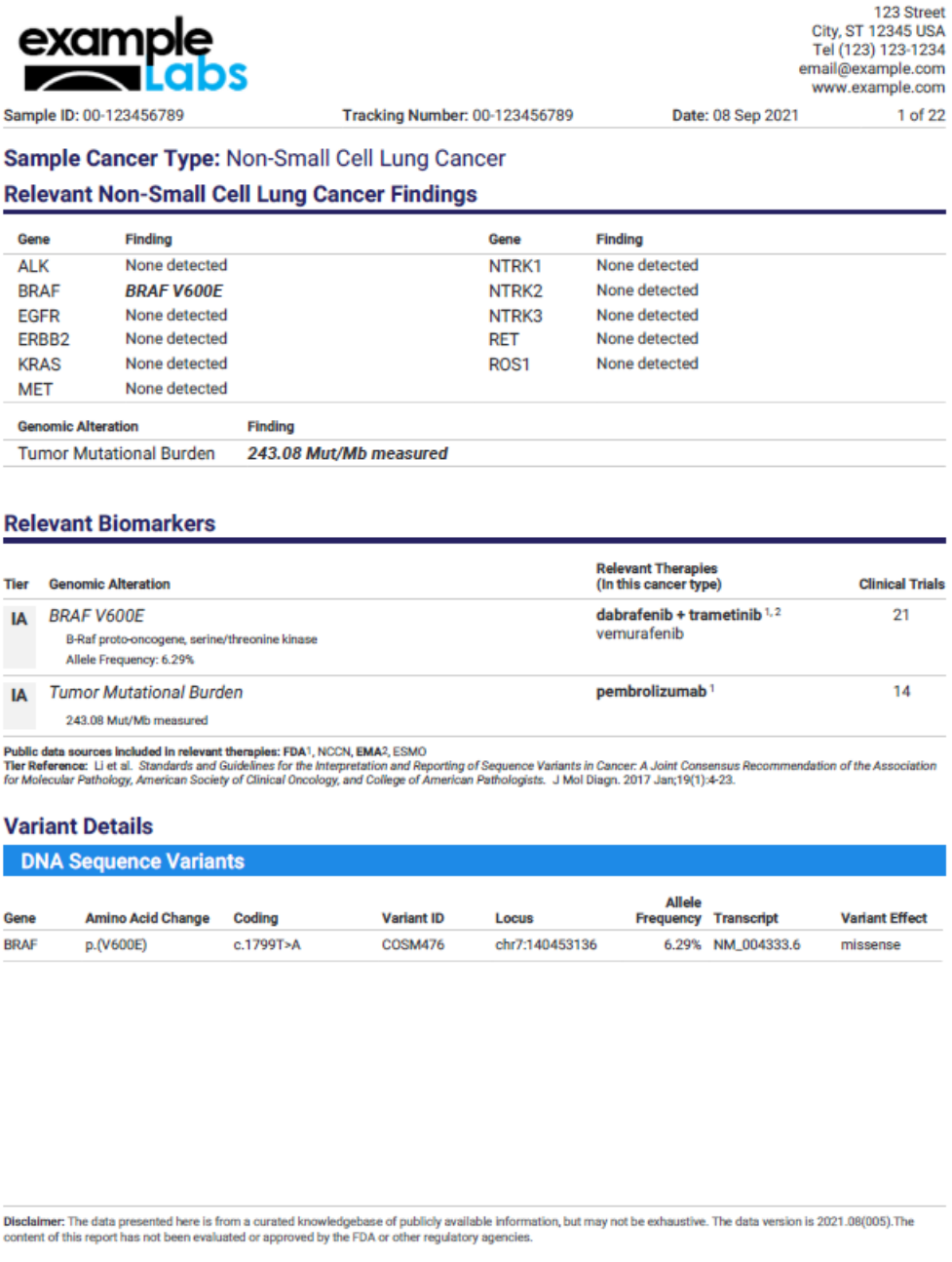
   Note: If you do not enter a filename, the software auto-generates a filename based on the VCF file uploaded: `<VCF filename>_OR.pdf`.

   c. Select whether to generate the report in grayscale.

   d. Select the report format: PDF or Text.

6. Click **Download Report** ⓓ, then save the report to your drive.

Oncomine™ Reporter example report



example Labs

123 Street
City, ST 12345 USA
Tel (123) 123-1234
email@example.com
www.example.com

Sample ID: 00-123456789 **Tracking Number:** 00-123456789 **Date:** 08 Sep 2021 1 of 22

Sample Cancer Type: Non-Small Cell Lung Cancer

Relevant Non-Small Cell Lung Cancer Findings

Gene	Finding	Gene	Finding
ALK	None detected	NTRK1	None detected
BRAF	***BRAF V600E***	NTRK2	None detected
EGFR	None detected	NTRK3	None detected
ERBB2	None detected	RET	None detected
KRAS	None detected	ROS1	None detected
MET	None detected		

Genomic Alteration	Finding
Tumor Mutational Burden	***243.08 Mut/Mb measured***

Relevant Biomarkers

Tier	Genomic Alteration	Relevant Therapies (In this cancer type)	Clinical Trials
IA	*BRAF V600E* B-Raf proto-oncogene, serine/threonine kinase Allele Frequency: 6.29%	**dabrafenib + trametinib** [1,2] vemurafenib	21
IA	*Tumor Mutational Burden* 243.08 Mut/Mb measured	**pembrolizumab** [1]	14

Public data sources included in relevant therapies: FDA[1], NCCN, **EMA**[2], ESMO
Tier Reference: Li et al. *Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer: A Joint Consensus Recommendation of the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists.* J Mol Diagn. 2017 Jan;19(1):4-23.

Variant Details

DNA Sequence Variants

Gene	Amino Acid Change	Coding	Variant ID	Locus	Allele Frequency	Transcript	Variant Effect
BRAF	p.(V600E)	c.1799T>A	COSM476	chr7:140453136	6.29%	NM_004333.6	missense

Disclaimer: The data presented here is from a curated knowledgebase of publicly available information, but may not be exhaustive. The data version is 2021.08(005).The content of this report has not been evaluated or approved by the FDA or other regulatory agencies.

Example of a solid tumor report

A Tips and 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™ Adhesive Film Applicator. Plate seals can be removed with less effort when hot. Try removing seals right after taking the plate out of the thermal cycler.
- Use IonCode™ Barcode Adapters to avoid handling and diluting adapters. Alternatively, combine and dilute Ion Xpress™ Barcode Adapters in large batches, then carefully aliquot into 96-well plates.
- If you are performing qPCR quantification, library amplification is unnecessary and the tube of Platinum™ PCR SuperMix High Fidelity supplied in the kit can be used for other applications. For more information, see the *Platinum™ PCR SuperMix High Fidelity User Guide*.
- If library yield is below 50 pM, libraries can still be sequenced by adding a proportionally larger volume to a combined library or template preparation.
- 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 Platinum™ PCR SuperMix HiFi and 3 µL of Library Amplification Primer Mix. Perform 5–10 library amplification cycles (see step 4 of "Amplify the library" on page 61 for cycling conditions).
- When amplifying multiple samples in a single PCR plate, ensure that the input across the samples is roughly equivalent so that the selected cycle number is optimal for all the samples in the run.

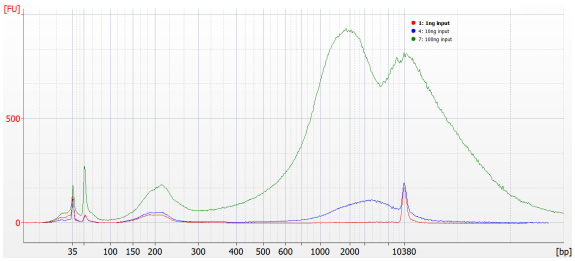
Troubleshooting

Low amplicon uniformity (DNA only)

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

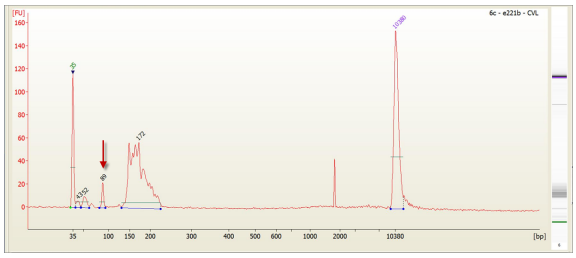
Library yield and quantification

Observation	Possible cause	Recommended action
Library concentration is low—general **Details:** (Library concentration is NOT indicative of quality.)	Sample DNA or RNA was mis-quantified.	Requantify sample DNA using the TaqMan™ RNase P Detection Reagents Kit; quantify RNA with a Qubit™ Fluorometer.
	Residual ethanol in sample DNA or RNA inhibited target amplification.	Incubate uncapped tube in hood for 1 hour.
		Speed-vac tube at room temperature for 5 minutes.
	Residual ethanol from AMPure™ purification inhibited library amplification.	Carefully remove all drops of ethanol before library amplification, then centrifuge plate, if necessary.
	Sample DNA or RNA quality was low.	Add more DNA/RNA or increase target amplification cycles.
	PCR, digestion, or ligation was inefficient.	Ensure proper dispensing and mixing of viscous components at each step.
	AMPure™ XP Beads were over-dried.	Do not dry the AMPure™ XP Beads more than 5 minutes.
	AMPure™ XP Beads inhibited library amplification.	Transfer library off of beads prior to amplification.
	qPCR cycling time is too short.	Use standard qPCR cycling for library designs >175 bp instead of Fast cycling.

Observation	Possible cause	Recommended action
Library concentration is too high	Sample DNA or RNA was mis-quantified.	Requantify sample DNA using the TaqMan™ RNase P Detection Reagents Kit; quantify RNA with a Qubit™ Fluorometer.
	More than 100 ng of sample DNA/RNA was used.	Add less DNA/RNA, or decrease target amplification cycles.
High molecular weight material is present on the Agilent™ 2100 Bioanalyzer™ instrument or library concentration is high on the Qubit™ Fluorometer  **Example Agilent™ 2100 Bioanalyzer™ analysis** showing presence of high molecular weight material.	High molecular weight DNA was not removed during purification of the amplified library (does not interfere with sequencing).	Remove less supernatant in the first-round (0.5X) purification and be sure not to disturb bead pellet.
		Increase AMPure™ XP Reagent volume from 25 µL (0.5X) to 35 µL (0.7X) in the first-round purification.
	Inserts are concatamerizing during the ligation step.	Reduce nucleic acid input amount.
		Requantify sample(s) with a Qubit™ Fluorometer.
		Reduce target amplification cycle number.
Library concentration is high as measured on the Agilent™ 2100 Bioanalyzer™ instrument	Markers are mis-assigned.	Ensure that markers are assigned correctly.

Other

Observation	Possible cause	Recommended action
The number of on-target reads is lower than expected	Unknown.	Increase the number of target amplification cycles by 2.
	Sample ID Panel targets were counted as off-target reads.	Add back the on-target reads from the Sample ID Panel.
Barcode representation is uneven (Equalizer™ kit not used)	Library quantification was inaccurate.	Use the Ion Library TaqMan™ Quantitation Kit for the most specific and accurate library quantification.
	Library combination was inaccurate.	Dilute libraries to 50 pM, then combine equal volumes.
Percentage of polyclonal ISPs is high (>40%)	Library input was too high.	Decrease amount of library added to the template preparation reaction by 50%.
	Library was mis-quantified.	Ensure that library was quantified accurately.

Observation	Possible cause	Recommended action
Percentage of polyclonal ISPs is high (>40%) *(continued)*	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.
Adapter dimers are present on the Agilent™ 2100 Bioanalyzer™ instrument at 90–105 bp or Adapter dimers are present during sequencing  **Adapter dimers.** Barcode adapters run at ≈53 bp, and barcode adapter dimers run at ≈105 bp.	Purification was inefficient.	In unamplified library purification, decrease AMPure™ XP Reagent volume from 45 µL (1.5X) to 30 µL (1X).
		In amplified library purification, decrease AMPure™ XP Reagent volume in second round from 60 µL (1.2X) to 50 µL (1.0X).
	Adapter dimers formed during reaction setup or during digestion.	Do not combine Adapters, DNA Ligase, and Switch Solution prior to addition.
		Use a 65°C temperature incubation instead of 60°C during the amplicon digestion step.
	Adapter concentration was too high.	Ensure that barcode adapters are diluted properly.

Chef-ready library preparation

For troubleshooting information when preparing Chef-ready libraries on an Ion Chef™ Instrument, see the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432).

B Supplemental information

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.

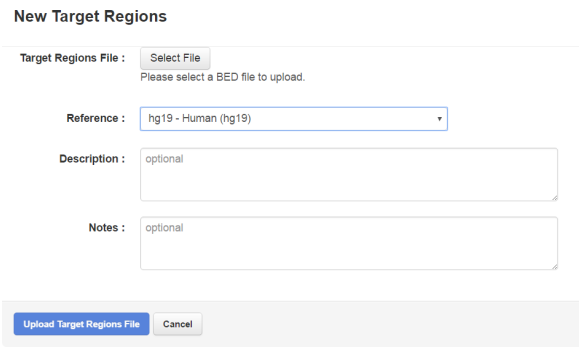
   Note: You can always change this selection in the Planned Run template workflow bar and in the Upload to IR quick link.

6. Click **Get Versions**, select **Ion Reporter 5.2 or later**, then click ✓**Add**.

Download and install BED files

Contact your field service representative to obtain the latest versions of Oncomine™ Focus 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_Focus.20160219.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.

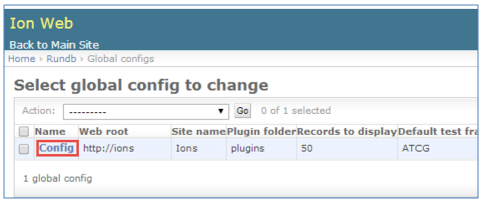
Make Oncomine™ Focus Assay templates available in Torrent Suite™ Software

The Oncomine™ templates are not enabled by default in Torrent Suite™ Software. Here we describe the administrator configuration step to enable these analysis templates. These instructions are intended for users who are familiar with the Admin Interface. Your ionadmin user account and password are required.

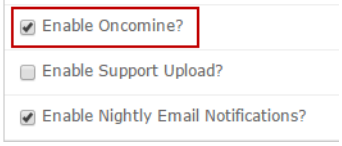
IMPORTANT! Changes in the admin interface can negatively impact the operation of your server. If you are ever in doubt, cancel your changes.

Follow these steps to enable Oncomine™ analyses on your system:

1. Log in to the Torrent Browser with your ***ionadmin*** account.
2. Click ⚙ ▸ **Configure**, scroll down to the **Database Administration** section, then click the **Admin Interface** link.
3. In the **Rundb** section click **Global configs**.
4. Click the **Config** link. (Your web root and site name are different.)



5. Scroll down to the bottom of the page. Check **Enable Oncomine?**, then click **Save**.



6. Click **Back to Main Site** to continue to work in Torrent Browser, or click **Log Out** to exit.

C

Quantify the amplified library with the Qubit™ Fluorometer

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

   Note:
   - The Platinum™ PCR SuperMix HiFi is used to elute the libraries from the beads.
   - The Platinum™ PCR SuperMix HiFi and Library Amplification Primer Mix can be combined before addition.

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

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

4. Seal the plate with MicroAmp™ Adhesive Film, place a MicroAmp™ Compression Pad on the plate, load in the thermal cycler, then run the following program:

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

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

Purify the amplified library

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

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

IMPORTANT!

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

First-round purification

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

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

Second-round purification

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

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

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

   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™ Clear 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" or
    - "Quantify library by qPCR and calculate dilution factor" on page 25

    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 (avg. 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 library to ~50 pM as described, combine, then proceed to template preparation, or store libraries as described below.

Store libraries

Libraries can be stored at 4–8°C for up to 1 month. For longer term, store at –20°C.

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

D

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	Publication No.
Oncomine™ Focus Assay User Guide	MAN0015819
Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide	MAN0013432
Ion Reporter™ Software 5.10 User Guide	MAN0017605
Torrent Suite™ Software 5.10 User Guide	MAN0017598
Oncomine™ Reporter User Guide	MAN0018068

Note: For additional documentation, see “Customer and technical support” on page 68.

Obtain information from the Help system

The Torrent Suite™ Software has a Help system that describes how to use each feature of the user interface.

In the toolbar of the Torrent Suite™ Software window, click **Help** ▸ **Software Help**.

You can use the Help system to find topics of interest by:

- Reviewing the table of contents
- Searching for a specific topic

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**.