

Oncomine™ Immune Response Research Assay USER GUIDE

Catalog Number A32881

Publication Number MAN0015867

Revision B.0



Manufacturer: Multiple Life Technologies Corporation manufacturing sites are responsible for manufacturing the products associated with the workflow covered in this guide.

Corporate entity: Life Technologies Corporation | Carlsbad, CA 92008 USA | Toll Free in USA 1 800 955 6288

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

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

Revision history: Pub. No. MAN0015867

Revision	Date	Description
B.0	31 May 2017	Include supplementary information for performing functional RNA quantification. Correction to the amount of 2X Oncomine™ Immune Response Research Panel–Chef Ready that is provided.
A.0	31 October 2016	<i>Oncomine™ Immune Response Research Assay User Guide</i> . Provides instruction for library preparation, templating, sequencing, and data analysis of Oncomine™ Immune Response Research Assay libraries.

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

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

©2017 Thermo Fisher Scientific Inc. All rights reserved.

Contents

■	CHAPTER 1	Product information	6
		Product description	6
		Contents and storage	7
		Oncomine™ Immune Response Research Assay	7
		Oncomine™ Immune Response Research Assay–Chef Ready	8
		Required materials not supplied	9
		Recommended materials	10
■	CHAPTER 2	Before you begin	11
		Procedural guidelines	11
		Before each use of the kit	11
		Workflow overview	12
■	CHAPTER 3	Library preparation	13
		Guidelines for RNA isolation and quantification	13
		Reverse transcription and target amplification	13
		Reverse transcribe the RNA	13
		<i>(Chef-ready kit only)</i> Transfer cDNA to an IonCode™ 96-well plate	14
		Prepare cDNA target amplification reactions	15
		Amplify the targets	16
		Partially digest amplicons	16
		Ligate adapters to the amplicons and purify	17
		Ion Xpress™ adapters only: Combine and dilute adapters	17
		Perform ligation reaction	18
		Purify the library	18
		Quantify the library by qPCR	20
		Elute the library	20
		Quantify the library by qPCR and calculate the dilution factor	20
		Store libraries	22
		Quantify the amplified library with the Qubit™ Fluorometer	23
		Amplify the library	23
		Purify the amplified library	23
		Qubit™ Fluorometer: Quantify the library and calculate the dilution factor	25
		Store libraries	26

Combine libraries	26
Guidelines for templating and sequencing	27
■ CHAPTER 4 Create an assay-specific Planned Run	28
About planned runs	28
Create a custom Planned Run template	29
Create a planned run	30
■ CHAPTER 5 Data analysis	32
Data analysis overview	32
Gene expression level quantification with the immuneResponseRNA plugin	32
Review run results	33
Sample Characteristics tab	34
Sample Correlation tab	37
Analysis of Selected Targets	38
Analysis of All Targets tab	39
Downloadable reports	40
Affymetrix™ Transcriptome Analysis Console (TAC) analysis	41
System requirements	41
Download and install TAC software	41
Perform TAC expression analysis	42
■ APPENDIX A Tips and troubleshooting	45
Tips	45
Troubleshooting	46
Library yield and quantification	46
Other	47
■ APPENDIX B Supplemental information	49
Functional RNA quantification	49
Install preloaded Reference Library file	52
Install Target Regions .bed files	52
Manually launch the immuneResponseRNA plugin	53
■ APPENDIX C Safety	55
Chemical safety	56
Biological hazard safety	57

Documentation and support 58

Obtain information from the Help system 58

Customer and technical support 58

Limited product warranty 58



Product information

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

Product description

The OncoMine™ Immune Response Research Assay is an RNA-based multi-biomarker next-generation sequencing (NGS) assay that enables the characterization of gene expression levels from approximately 400 genes relevant to oncology and immune therapy response research.

This guide covers the following products:

- OncoMine™ Immune Response Research Assay (Cat. No. A32881)
- Ion AmpliSeq™ Library Kit 2.0 (Cat. No. 4475345)
- SuperScript™ VILO™ cDNA Synthesis Kit (Cat. No. 11754050)
- IonCode™ Barcode Adapters (Cat. No. A29751)
- Ion Xpress™ Barcode Adapters (various Cat. Nos.)
- Ion AmpliSeq™ Sample ID Panel (Cat. No. 4479790)

SuperScript™ and Ion AmpliSeq™ products include reagents to prepare targeted libraries from RNA for sequencing on the Ion S5™/Ion S5™ XL or Ion PGM™ Systems. The kits are designed for use with barcoded adapters so that multiple libraries can be combined and loaded onto a single Ion Chip to minimize the per-sample sequencing cost.

Note: The OncoMine™ Immune Response Research Assay–Chef Ready library preparation kit (Cat. No. A32928) is also available for automated library preparation (see “OncoMine™ Immune Response Research Assay–Chef Ready” on page 8). The kit provides OncoMine™ Immune Response Research Panel–Chef Ready at 2X concentration pre-measured in barcoded Primer Pool tubes ready to load into an Ion AmpliSeq™ Chef Reagents DL8 cartridge.

Contents and storage

Oncomine™ Immune Response Research Assay

The Oncomine™ Immune Response Research Assay (Cat. No. A32881) consists of the Oncomine™ Immune Response Research Panel and the Ion AmpliSeq™ Library Kit 2.0 for the rapid preparation of barcoded sample libraries from RNA, which you can then combine and sequence simultaneously.

Contents	Amount	Storage
Oncomine™ Immune Response Research Assay (Part No. A31930)		
5X Oncomine™ Immune Response Research Panel	4 × 32 µL	-25°C to -15°C
Ion AmpliSeq™ Library Kit 2.0 (Part No. 4475345)		
5X Ion AmpliSeq™ HiFi Mix (red cap)	32 µL	-30°C to -10°C
FuPa Reagent (brown cap)	16 µL	
Switch Solution (yellow cap)	32 µL	
DNA Ligase (blue cap)	16 µL	
Ion AmpliSeq™ Adapters (green cap)	16 µL	
Platinum™ PCR SuperMix HiFi (black cap)	400 µL	
Library Amplification Primer Mix (white cap)	16 µL	
Low TE (clear cap)	1.7 mL	15°C to 30°C ^[1]

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

**Oncomine™
Immune Response
Research Assay–
Chef Ready**

The Oncomine™ Immune Response Research Assay–Chef Ready Kit (Cat. No. A32928, ordered separately) provides Oncomine™ Immune Response Research Panel–Chef Ready 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. See the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432) for detailed information on preparing Oncomine™ Immune Response Research Assay libraries on the Ion Chef™ System.

Component	Quantity per kit	Storage
Oncomine™ Immune Response Research Panel–Chef Ready (Part. No. A32929)		
2X Oncomine™ Immune Response Research Panel–Chef Ready	8 × 150 µL	–30°C to –10°C
Ion AmpliSeq™ Kit for Chef DL8 (Cat. No. A29024)		
Ion AmpliSeq™ Chef Reagents DL8 (Part No. A29025)	4 cartridges	–30°C to –10°C
Ion AmpliSeq™ Chef Solutions DL8 (Part No. A29026)	4 cartridges	2°C to 8°C ^[1]
Ion AmpliSeq™ Chef Supplies DL8 (per insert) (Part No. A29027) <ul style="list-style-type: none"> • Ion AmpliSeq™ Tip Cartridge L8 • PCR Frame Seal • Enrichment Cartridge 	1 box with 4 inserts	15°C to 30°C
IonCode™ 0101–0132 in 96 Well PCR Plates (dried) (Part No. A29028) <p>Set includes 4 PCR plates:</p> <ul style="list-style-type: none"> • IonCode™ 0101–0108 in 96 Well PCR Plate (red) • IonCode™ 0109–0116 in 96 Well PCR Plate (yellow) • IonCode™ 0117–0124 in 96 Well PCR Plate (green) • IonCode™ 0125–0132 in 96 Well PCR Plate (blue) 	1 set of 4 plates	15°C to 30°C

^[1] Ion AmpliSeq™ Chef Solutions DL8 cartridges are shipped at ambient temperature, but 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: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
SuperScript™ VIL0™ cDNA Synthesis Kit	11754050
IonCode™ Barcode Adapters 1–384 Kit	A29751
OR	
Ion Xpress™ Barcode Adapters	4471250
Agencourt™ AMPure™ XP Kit	Beckman Coulter, A63880 or A63881
One of the following: <ul style="list-style-type: none"> • GeneAmp™ PCR System 9700 or Dual 96-well Thermal Cycler • AB™ 2720 Thermal Cycler • Veriti™ 96-well Thermal Cycler • ProFlex™ 96-well PCR System 	See web product pages
MicroAmp™ Optical 96-well Reaction Plate	N8010560 4306737 (with barcode)
MicroAmp™ Fast Optical 96-Well Reaction Plate	4346907
MicroAmp™ Optical Adhesive Film	4311971
MicroAmp™ Adhesive Film	4306311
MicroAmp™ Compression Pad	4312639
DynaMag™ -96 Side Magnet, or other plate magnet	12331D
Non-stick RNase-Free Microfuge Tubes	MLS
Low TE buffer	MLS
Nuclease-free Water	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: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Recommended additional equipment	
Real-time PCR instrument (e.g., Applied Biosystems™ 7900HT, 7500, StepOnePlus™, ViiA™ 7 Systems, or a QuantStudio™ Real-Time PCR System)	See web product pages
96-well plate centrifuge	MLS
Recommended for nucleic acid isolation	
RecoverAll™ Multi-Sample RNA/DNA Workflow	A26069
RecoverAll™ Total Nucleic Acid Isolation Kit	AM1975
Recommended for library quantification	
Ion Library TaqMan® Quantitation Kit	4468802
Qubit™ 3.0 Fluorometer or Qubit™ 2.0 Fluorometer ^[1] and Qubit™ dsDNA HS Assay Kit (DNA)	Q33216, Q32851/Q32854
Recommended for functional RNA quantification	
Promyelocytic Leukemia (HL-60) Total RNA	AM7836
TaqMan® Fast Virus 1-Step Master Mix	4444432
TaqMan® Gene Expression Assay, GUSB (20X, Hs00939627)	4331182

^[1] Supported but no longer available for purchase.



Before you begin

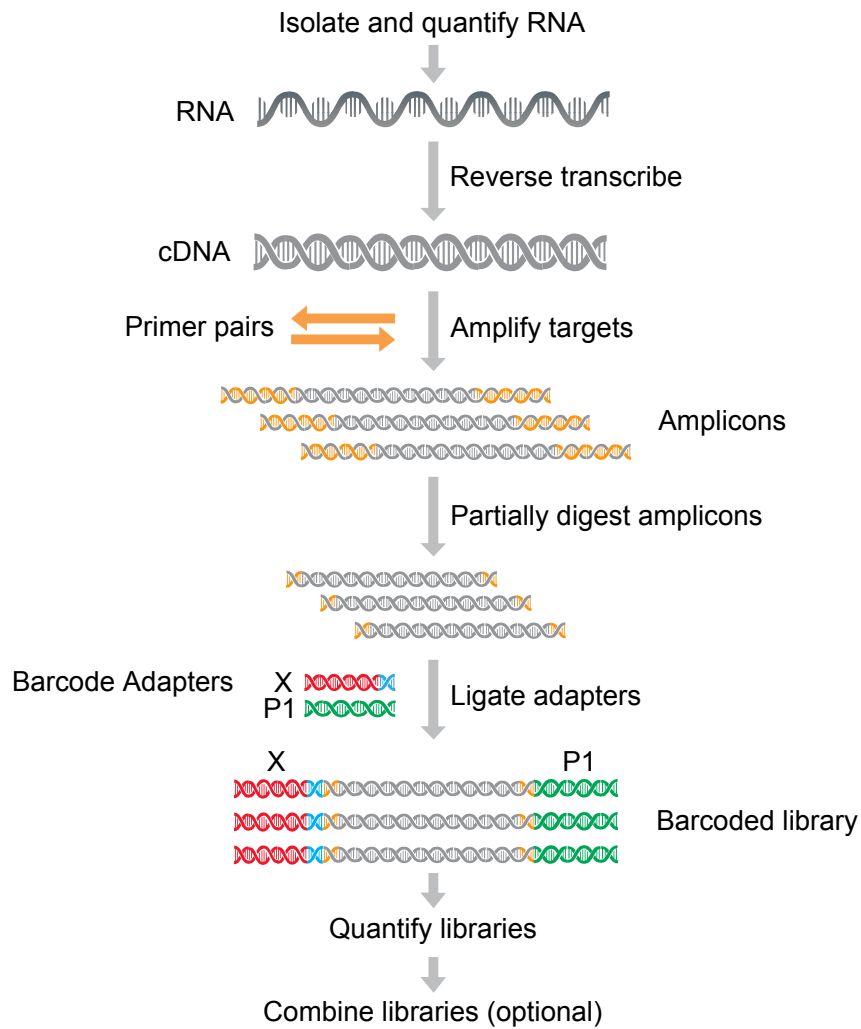
Procedural guidelines

- Minimize freeze-thaw cycles of Oncomine™ Immune Response Research Assay panels by aliquoting as needed for your experiments. Panels can be stored at 4°C for one year.
- Use good laboratory practices to minimize cross-contamination of products. If possible, perform PCR setup in an area or room that is free of amplicon contamination. Always change pipette tips between samples.
- Use a calibrated thermal cycler specified in “Required materials not supplied”.
- Pipet viscous solutions, such as 5X Ion AmpliSeq™ HiFi Mix, FuPa Reagent, Switch Solution, DNA Ligase, and panels, slowly and ensure complete mixing by vortexing or pipetting up and down several times.

Before each use of the kit

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

Workflow overview



3

Library preparation

IMPORTANT! We recommend Oncomine™ Immune Response Research Assay – Chef Ready Kit (Cat. No. A32928) users perform the reverse transcription as described in the following section “Reverse transcribe the RNA”. Following completion of cDNA synthesis refer to the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432) for instructions to prepare Oncomine™ Immune Response Research Assay libraries on the Ion Chef™ System.

Guidelines for RNA isolation and quantification

- Use kits in “Recommended materials” on page 10 for isolating total RNA.
- We recommend verifying sample RNA integrity by performing the functional RNA quantification, see page 49 for more information.
- 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/ μ L).
- 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.

Reverse transcription and target amplification

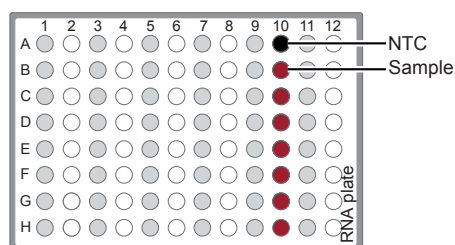
Reverse transcribe the RNA

1. If the RNA was prepared from FFPE tissue and not previously heat-treated, heat at 80°C for 10 minutes, then cool to room temperature.
2. If there is visible precipitate in the 5X VILO™ Reaction Mix tube or cap after thawing, vortex or pipet up and down at room temperature to resuspend before pipetting.

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

Component	Volume
5X VILO™ Reaction Mix	2 µL
10X SuperScript™ Enzyme Mix	1 µL
Total RNA (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 non-template control (NTC).



4. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least five times before sealing the plate.
5. Place a MicroAmp™ Compression Pad on the plate, load the plate in the thermal cycler, then run the following program to synthesize cDNA.

Temperature	Time
25°C	10 minutes
42°C	60 minutes
85°C	5 minutes
4°C	Hold

STOPPING POINT Samples can be stored at 4°C for up to 16 hours in the thermal cycler. For longer term, store at -20°C.

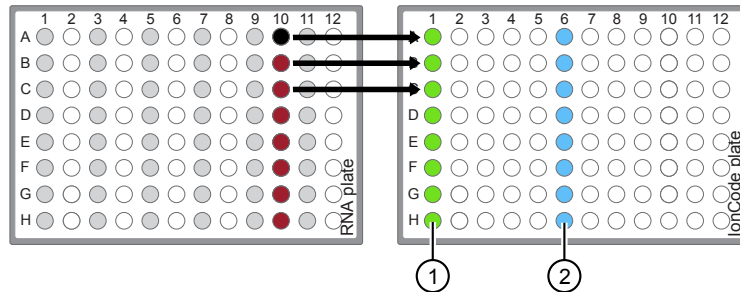
6. Centrifuge the plate briefly to collect any droplets at the bottom of the wells, then proceed to the next step.

(Chef-ready kit only) Transfer cDNA to an IonCode™ 96-well plate

Oncomine™ Immune Response Research Assay (Cat. No. A32881) users performing manual library preparation proceed to “Prepare cDNA target amplification reactions”, without transferring the cDNA synthesis reactions.

1. Place the 96-well plate in a pre-chilled cold block.
2. Remove the seal from the plate, then transfer each individual cDNA synthesis reaction to the corresponding row in column 1 of an IonCode™ 96-well plate.

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



- Each column 1 well contains 10- μL cDNA synthesis reaction, and 5- μL nuclease-free water.
 - Each column 6 well contains a dried-down IonCode™ barcode. Lowest barcode number is in A6 and the highest is in H6. All appear light blue in the actual plate.
- 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.

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).

Information required to set up the Ion Chef™ Instrument run is in the following table.

# of primer pools	Target amplification cycles	Anneal & extension time
1	20	4 minutes

Prepare cDNA target amplification reactions

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

- Place the 96-well plate in a pre-chilled cold block or on ice.
- Thaw the 5X Ion AmpliSeq™ HiFi Mix on ice, gently vortex to mix, then centrifuge briefly to collect.
- 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™ Immune Response Research Panel	4 μL
Nuclease-free Water	2 μL
Total volume per well (includes 10 μL from cDNA synthesis)	~20 μL

- 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.

Proceed to “Amplify the targets”.

Amplify the 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 the enzyme	99°C	2 min
19 cycles	Denature	99°C	15 sec
	Anneal and extend	60°C	4 min
Hold	—	10°C	Hold

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.

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

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

Partially digest amplicons

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

1. Keep the FuPa Reagent (brown cap) on ice, gently vortex to mix, then briefly centrifuge to collect.
2. Carefully remove the adhesive film from the plate.

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

3. Add 2 µL of FuPa Reagent to each amplified sample. The total volume is ~22 µL.
4. Seal the plate with a new clear adhesive film, vortex thoroughly, then centrifuge briefly to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

5. 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)

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

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™
adapters only:
Combine and
dilute adapters**

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

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

^[1] X = barcode chosen

Note: Store diluted adapters at -20°C.

Perform 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 pipeting.
2. Carefully remove the plate seal, then add the following components in the order listed to each well containing digested amplicons.

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 μ L
2	Ion Xpress™ barcode adapter mix	2 μ L
—	Total volume (including ~22 μ L of digested amplicon)	~28 μL

3. Seal the plate with a new MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.
4. Carefully remove the plate seal, then add 2 μ L of DNA Ligase (blue cap) to each well.
5. Seal the plate with a new film, vortex thoroughly, then briefly centrifuge to collect droplets.
6. Place a MicroAmp™ Compression Pad on the plate, load the plate in the thermal cycler, then run the following program:

Temperature	Time
22°C	30 minutes
72°C	10 minutes
10°C	Hold (for up to 60 minutes)

STOPPING POINT Samples can be stored for longer periods 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 μ L \times # 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 μ L (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 “Quantify the library by qPCR” on page 20

Note: Alternatively, quantify the libraries using a Qubit™ 2.0 or 3.0 Fluorometer, see “Quantify the amplified library with the Qubit™ Fluorometer” on page 23.

Quantify the library by qPCR

Elute the library, then determine the concentration by qPCR with the Ion Library TaqMan® Quantitation Kit (Cat. No. 4468802). Libraries that have not undergone a second round of amplification typically have yields of 100–500 pM. However, yield is not indicative of library quality. After quantification, determine the dilution factor that results in a concentration of ~50 pM.

Elute the library

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

Quantify the library by qPCR and calculate the dilution factor

Determine the concentration of each Oncomine™ Immune Response Research Assay library by qPCR with the Ion Library TaqMan® Quantitation Kit using the following steps. Analyze each sample, standard, and negative control in duplicate reactions.

1. Prepare three 10-fold serial dilutions of the *E. coli* DH10B Ion Control Library (~68 pM; from the Ion Library TaqMan® Quantitation Kit) at 6.8 pM, 0.68 pM, and 0.068 pM. Mark these tubes as standards, then use these concentrations in the qPCR instrument software.
2. Calculate, then prepare the required volume of PCR master mix for duplicate reactions of each library sample, standard, and NTC using the following table. Include a 5–10% overage to accommodate pipetting errors.

Component	Volume per reaction	
	96-well plate	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, set up duplicate PCR reactions for each sample, standard, and NTC. To each well add the following components:

Component	Volume per reaction	
	96-well plate	384-well plate
PCR 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 follows:

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

- Enter the concentrations of the control library standards.
- Select ROX™ Reference Dye as the passive reference dye.
- Select a reaction volume of 20 µL.
- Select FAM™ dye/MGB as the TaqMan® probe reporter/quencher.

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™ Immune Response Research 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 Ion Chef™ template preparation.
 - ~100 pM for Ion OneTouch™ 2 template preparation.

For example (Ion Chef™ template preparation):

- The undiluted library concentration is 300 pM.
 - The dilution factor is $300 \text{ pM}/50 \text{ pM} = 6$.
 - Therefore, 10 μL of library that is mixed with 50 μL of Low TE (1:6 dilution) yields approximately 50 pM.
7. Dilute the library as described, then proceed to template preparation, or combine 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” for cycling conditions).

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.

Quantify the amplified library with the Qubit™ Fluorometer

Oncomine™ Immune Response Research Assay libraries must be amplified and purified to obtain sufficient material for accurate quantification using the Qubit™ 2.0 or 3.0 Fluorometer. Amplified libraries typically have yields of 2,000–10,000 pM. After quantification, determine the dilution factor that results in a concentration of 40–60 pM, which is appropriate for template preparation using the Ion Chef™ System.

Note: Library yield is not indicative of library quality.

Amplify the library

1. Remove the plate with purified libraries from the plate magnet, then **add 50 µL of Platinum™ PCR SuperMix HiFi and 2 µL of Library Amplification Primer Mix** to each bead pellet.

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

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

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

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

Purify the amplified library

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

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

IMPORTANT!

- Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.
 - Use freshly prepared 70% ethanol for the next steps. Combine 230 µL of ethanol with 100 µL of Nuclease-free Water per sample.
 - Do **NOT** substitute a Dynabeads™-based purification reagent for the Agencourt™ 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. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a magnet such as the DynaMag™ Side Magnet for at least 5 minutes or until the solution is clear.
5. Carefully transfer the supernatant from each well to a new well of the 96-well PCR plate without disturbing the pellet.

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

Second-round purification

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

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

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

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

5. Repeat step 4 for a second wash.
6. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 2–5 minutes. **Do not overdry.**

7. Remove the plate from the magnet, then **add 50 µL of Low TE** to the pellet to disperse the beads.
8. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge to collect droplets. Alternatively, mix by setting a pipettor to 40 µL, then pipet the mixture up and down at least 5 times before sealing the plate.
9. Incubate at room temperature for at least 2 minutes.
10. Place the plate in the magnet for at least 2 minutes, then analyze an aliquot of the supernatant as described in:
 - “Quantify the library by qPCR and calculate the dilution factor” or
 - “Qubit™ Fluorometer: Quantify the library and calculate the dilution factor”

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 the Qubit™ 2.0 or 3.0 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™ 2.0 or Qubit™ 3.0 Fluorometer.
 - e. Calculate the concentration of the undiluted library by multiplying by 20.
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 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.

Combine libraries

Multiple barcoded Oncomine™ Immune Response Research Panel libraries can be sequenced on a single chip by combining equal volumes of each library before template preparation. We recommend combining up to a maximum of 8 libraries for the following Ion sequencing chips:

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

Ion sequencing chip	Maximum number of libraries (X)
Ion 318™ Chip v2	4
Ion 520™ Chip	4
Ion 530™ Chip	8

Prepare a combined library as follows.

1. Dilute all individual barcoded libraries to 50 pM concentration.
2. Combine 10 µL of each library in a single 1.5-mL Eppendorf LoBind™ tube.
3. After adding the last library, pipet up and down 5 times to mix, then centrifuge briefly to collect in the bottom of the tube.

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

Proceed to templating and sequencing, see “Guidelines for templating and sequencing” on page 27 for more information.

Guidelines for templating and sequencing

Proceed to template preparation and sequencing using the following kits appropriate to your instrument setup. To create a specific Run Plan for use in templating and sequencing see Chapter 4, “Create an assay-specific Planned Run”. Refer to the appropriate user guide or guides listed in the table for more information.

Template System	Sequencer	Kit	User Guide
OT2	PGM	Ion PGM™ Hi-Q™ View OT2 Kit (Cat. No. A29900)	<i>Ion PGM™ Hi-Q™ View OT2 Kit User Guide</i> (Pub. No. MAN0014579)
		<i>and</i>	<i>and</i>
		Ion PGM™ Hi-Q™ View Sequencing Kit (Cat. No. A30044)	<i>Ion PGM™ Hi-Q™ View Sequencing Kit User Guide</i> (Pub. No. MAN0014583)
	S5	Ion 520™ & Ion 530™ Kit – OT2 (Cat. No. A27751)	<i>Ion 520™ & Ion 530™ Kit – OT2 User Guide</i> (Pub. No. MAN0010844)
Chef	PGM	Ion PGM™ Hi-Q™ View Chef Kit (Cat. No. A29902)	<i>Ion PGM™ Hi-Q™ View Chef Kits User Guide</i> (Pub. No. MAN0014571)
	Ion S5	Ion 520™ & Ion 530™ Kit – Chef (Cat. Nos. A27757, A30010)	<i>Ion 520™ & Ion 530™ Kit – Chef User Guide</i> (Pub. No. MAN0010846)



Create an assay-specific Planned Run

IMPORTANT! This kit is compatible with Torrent Suite™ Software 5.2.1 or later. Before proceeding, check for updates to the Torrent Suite™, and Ion Chef™ System software, and install the updates if available.

About planned runs

Planned Runs contain all the settings used in a sequencing run, including number of flows, kit types, barcodes, sample information, and reference files (if any). Planned Runs are used to track samples, chips, and reagents throughout the sequencing workflow, from template preparation through sequencing and subsequent data analysis.


In Torrent Suite™ Software two Planned Run templates for use with the Oncomine™ Immune Response Research Assay are provided.

Application	Torrent Suite™ Software template	Description
Ion AmpliSeq™ RNA	Oncomine™ Immune Response Research Assay for S5 with Chef	RNA-only planned run template for automated library preparation, template preparation, and sequencing using the Ion Chef™ and Ion S5™ Systems.
	Oncomine™ Immune Response Research Assay for PGM with OT2	RNA-only planned run template for template preparation and sequencing using the Ion OneTouch™ 2 and Ion PGM™ Systems.

IMPORTANT! The pre-loaded Run Plan template "Oncomine™ Immune Response Research Assay for S5 with Chef " is configured for automated library preparation using the Ion AmpliSeq™ Kit for Chef DL8 and IonCode™ Barcode Adapters. See "Create a custom Planned Run template" to create a Run Plan template for use with the Oncomine™ Immune Response Research Assay (Cat. No. A32881) that includes the Ion AmpliSeq™ Library Kit 2.0 and uses Ion Xpress™ or IonCode™ Barcode Adapters for library preparation.

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 *Torrent Suite User Documentation* available through the **Help ▶ Software Help** menu in the Torrent Browser software.

1. Sign in to the Torrent Browser for the Torrent Server connected to your Ion Chef™ System.
2. Under the **Plan** tab, in the **Templates** screen, click **AmpliSeq RNA** in the left navigation menu.
3. In the **AmpliSeq RNA** list, find the **Template Name** that corresponds to your instrumentation set up, click  then select **Copy**.

Instrumentation set up	Template
Ion OneTouch™ 2 System/Ion PGM™ Sequencer	Oncomine Immune Response Research Assay for PGM with OT2
Ion Chef™ System/Ion S5™ or Ion S5™ XL Sequencer	Oncomine Immune Response Research Assay for S5 with Chef

The copy template wizard will open to the **Save** tab.

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

Field	Action...
Template Name	Enter a name for the Planned Run template.
Reference Library ^[1]	Select ImmuneResponse_v3.1
Target Regions ^[2]	Select ImmuneResponse_v3.1_target_designed_20160908.bed

^[1] Prior to creating your first planned run, install the preloaded Reference Library file. See "Install preloaded Reference Library file" on page 52 for instructions.

^[2] Contact your field service representative to obtain the most up to date Target Regions .bed file. See page 52 for instructions to install the Target Regions BED file.

5. In the **Application** tab, verify that **RNA** and **AmpliSeq RNA** are selected for **Application** and **Target Technique** respectively, then click **Next**.
6. In the **Kits** page, select the **Ion Chef Template Kit** radio button, then complete the following fields:

Field	Selection
Instrument	Ion S5™ System
Library Kit Type	Ion AmpliSeq™ Library Kit 2.0 ^[1]
Template Kit	Ion 520™ & Ion 530™ Kit – Chef
Sequencing Kit	Ion S5™ Sequencing Kit
Base Calibration Mode	Default Calibration

Field	Selection
Chip Type	Ion 530™ Chip
Barcode Set	Ion Xpress™ ^[2]
Flows	500



^[1] Select **Ion AmpliSeq Kit for Chef DL8** if using (Cat. No. A32928).

^[2] Select **IonCode** if using (Cat. No. A32928).

7. Select or edit the optional information fields appropriately for your run, then click **Next**.
8. In the **Plugins** tab, ensure **immuneResponseRNA** is selected, then click **Configure**.
9. Deselect **Filter Barcodes** and **ERCC Tracking**, click **Save Changes**, then click **Next**.
10. Review the **Projects** tab and make selections appropriate to your run, then click **Next**.
11. In the **Save** tab, click **Copy Template** to save the new run template.

The customized template is now available in the **AmpliSeq RNA** page.

Create a planned run

1. Sign in to the Torrent Browser for the Torrent Server connected to your Ion Chef™ System.
2. Under the **Plan** tab, in the **Templates** screen, click **AmpliSeq RNA** in the left navigation menu.
3. In the AmpliSeq RNA list, click on your customized Planned Run template name, alternatively click  ▼ then select **Plan Run**.
The create plan wizard will open to the **Plan** tab.
4. Enter a **Run Plan Name**.
5. Verify the **Use same reference & BED files for all barcodes** radio button is selected.
6. In the **Number of barcodes** field, enter the number of barcodes that will be used in this run, then click the check mark  button to the right of this field.
7. In the **Sample Tube Label** field(s), enter or scan the barcode of the Ion Chef™ Library Sample Tube that will be used in the run.

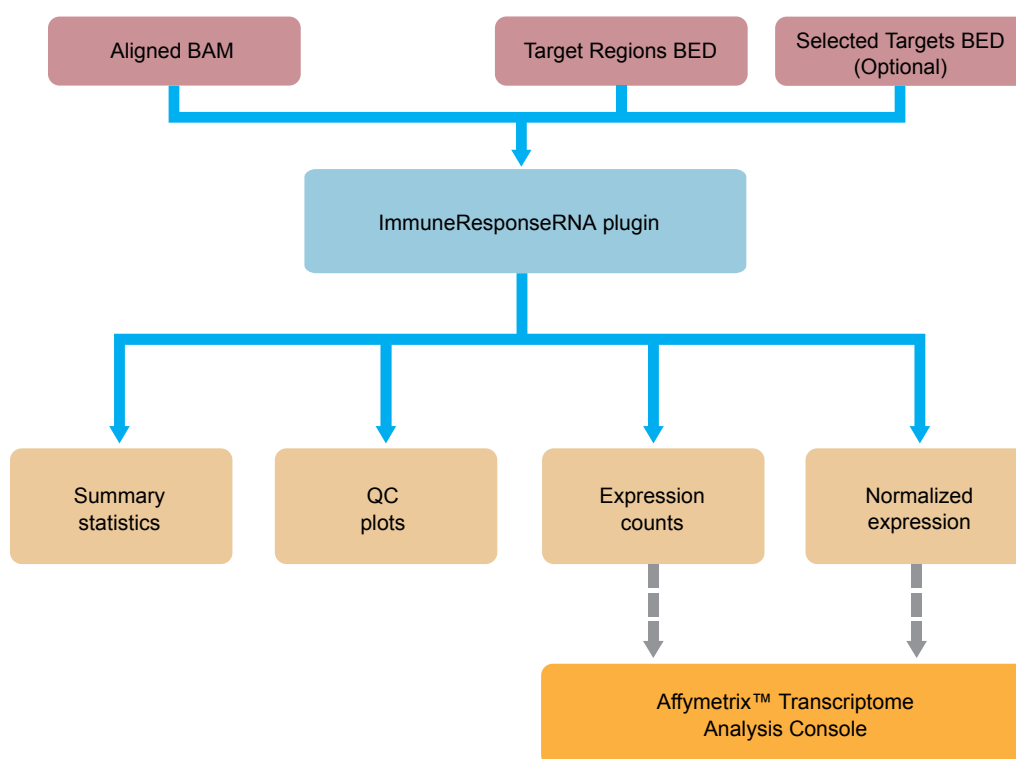
8. For each sample select the **Barcode** that will identify it, then enter or select from the available dropdown list the appropriate information for each field (all fields are required except **Sample Description** and **Sample ID**).

IMPORTANT! **Sample Names** must be unique to each sample. Do not duplicate sample names.

9. Click **Plan Run**.

The run is listed in the **Planned Run List** page 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.

Data analysis overview



Gene expression level quantification with the immuneResponseRNA plugin

Gene expression level quantification for the OncoPrint™ Immune Response Research Assay is performed with the Torrent Suite™ immuneResponseRNA plugin that produces gene transcript quantification from sequence read data.

The immuneResponseRNA plugin requires a **Target Regions** (.bed) file and an associated **Reference Library** (.fasta) file (see “Install Target Regions .bed files” on page 52 for more information on installing these files). The plugin also accepts a second—optional—.bed file that specifies a subset of target genes allowing sample clustering. To automatically analyze the data on completion of an OncoPrint™ Immune Response Research Assay sequencing run, select the immuneResponseRNA plugin when creating the Run Plan. Alternatively, the plugin analysis can be launched

manually following completion of a sequencing run (see “Manually launch the immuneResponseRNA plugin” on page 53 for more information). The resultant immuneResponseRNA Report includes gene expression counts (number of aligned reads to a given gene target), a data analysis summary, and QC plots. The normalized, gene-level count data from the run are available for download for further analyses with Affymetrix™ Transcriptome Analysis Console (TAC) 3.1 software.

Review run results

After your sequencing run completes, review the results in the **Run Summary** page.

1. Click the **Data tab** ▶ **Completed Runs & Results**, then search for your run.
2. Click the **Report Name** link to open the **Run Summary** page for your run.
3. Scroll down to the **immuneResponseRNA** section, then click the **immuneResponseRNA.html** link to open the **immuneResponseRNA Report** for all barcodes. Alternatively, click an individual barcode name to view the results for that barcode.

immuneResponseRNA (v5.2.1.2) [immuneResponseRNA.html](#) Completed ▾

Target regions: ImmuneResponse_v3.1_target_designed_20160908
Genes of interest region: None
Read filters: Alignment length at least 50% of insert size.
Time of analysis: 2016-10-25 14:43

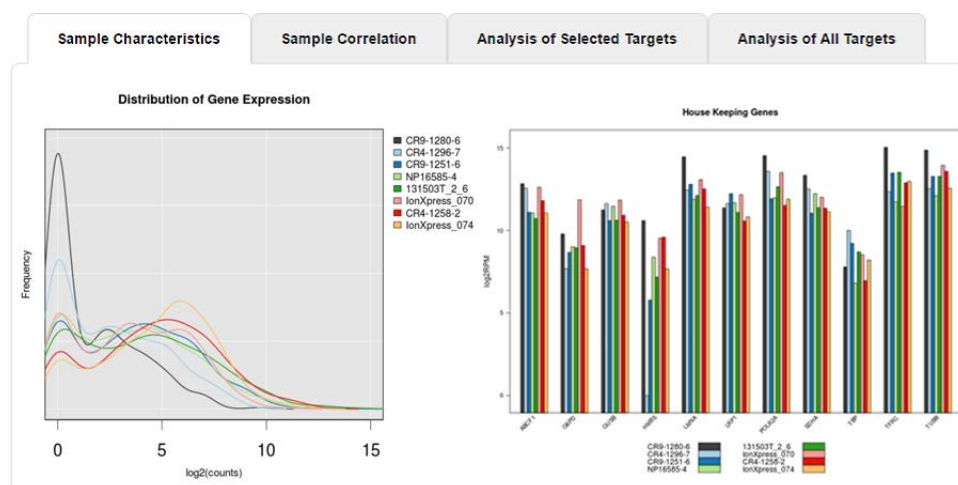
Barcode Name	Sample	Mapped Reads	Valid Reads	Targets >= 1 reads	Targets >= 2 reads	Targets >= 10 reads
IonCode_0101	NSCLC_LL	9,860	77.71%	274	247	131
IonCode_0102	NSCLC_F	70,616	96.33%	334	318	237
IonCode_0103	NSCLC_I	84,924	94.74%	332	313	241
IonCode_0104	NSCLC_C	71,027	95.28%	346	318	243
IonCode_0105	NSCLC_B	54,941	92.44%	347	333	272
IonCode_0106	NSCLC_NN	1,681	93.58%	124	92	41
IonCode_0107	NSCLC_J	73,573	93.97%	333	312	242
IonCode_0108	NSCLC_DD	53,836	94.59%	301	278	194

◀ 1 ▶ 10 items per page 1 - 8 of 8 items

4. In the **Analysis Summary**, review your **Mapped Reads**, **Valid Reads**, and **Targets** detected by barcode.

Column	Description
Barcode Name	The barcode used for the sample.
Sample	Sample name as it was entered in the sequencing Run Plan.
Mapped Reads	Number of reads that map to the reference sequences.
Valid Reads	Percentage of mapped reads $\geq 50\%$ amplicon length.
Targets ≥ 1 reads	Number of targets/genes with at least 1 read.
Targets ≥ 2 reads	Number of targets/genes with at least 2 reads.
Targets ≥ 10 reads	Number of targets/genes with at least 10 reads.

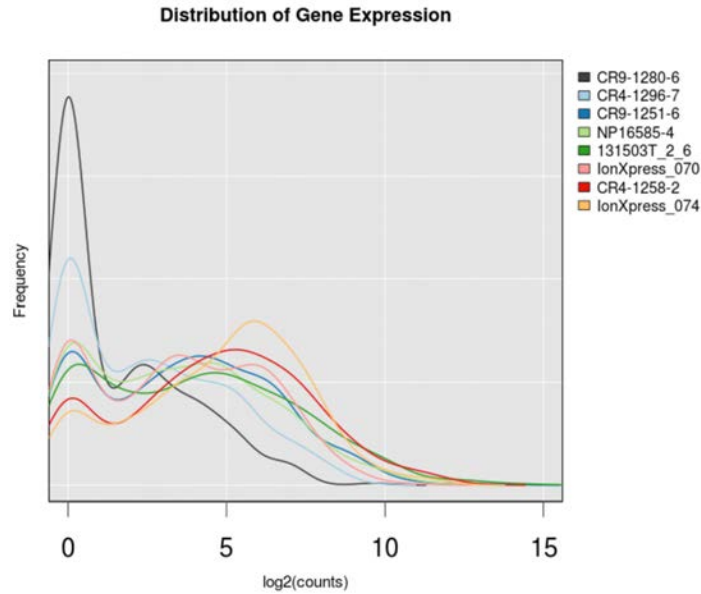
5. Scroll down then click the **Sample Characteristics**, **Sample Correlation**, **Analysis of Selected Targets** (only present if a **Genes of interest** subset .bed file was selected), or **Analysis of All Targets** tabs to review the data graphically.



Sample Characteristics tab

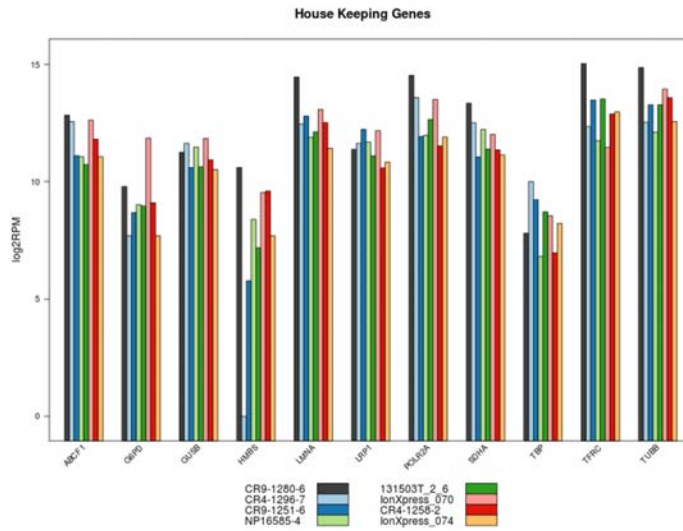
The Sample Characteristics tab contains 4 plots:

Distribution of Gene Expression



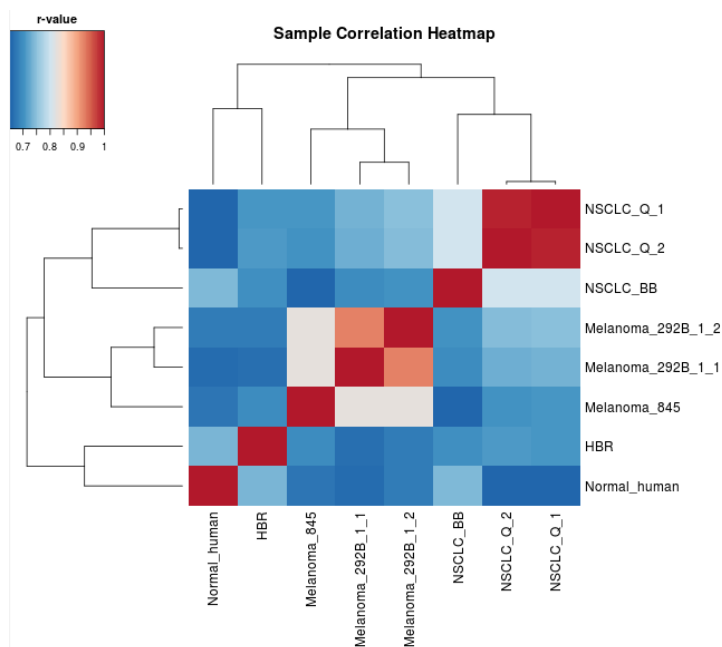
This plot shows the density distribution (similar to a histogram) of gene expression ($\log_2(\text{counts} + 1)$). The plot is created by fitting the count data to a Gaussian kernel using the default R 'density' function.

Housekeeping Genes



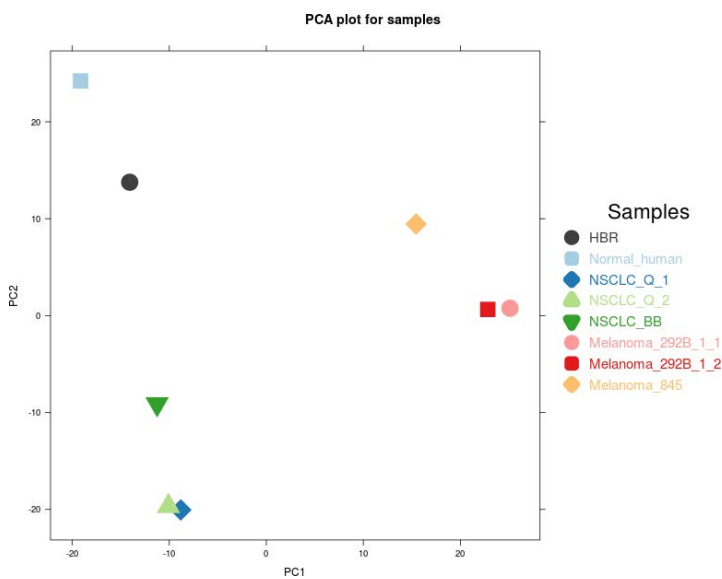
This plot shows the expression ($\log_2 \text{RPM} + 1$, RPM: read per million of mapped reads) of 11 housekeeping genes. These genes are used to compute the normalization factor. Colors specify barcoded samples.

Sample Correlation Heatmap



A heatmap plot created with heatmap.2 function in R package gplots. The dendrogram reflects the clustering of barcoded samples measured using correlation values between samples.

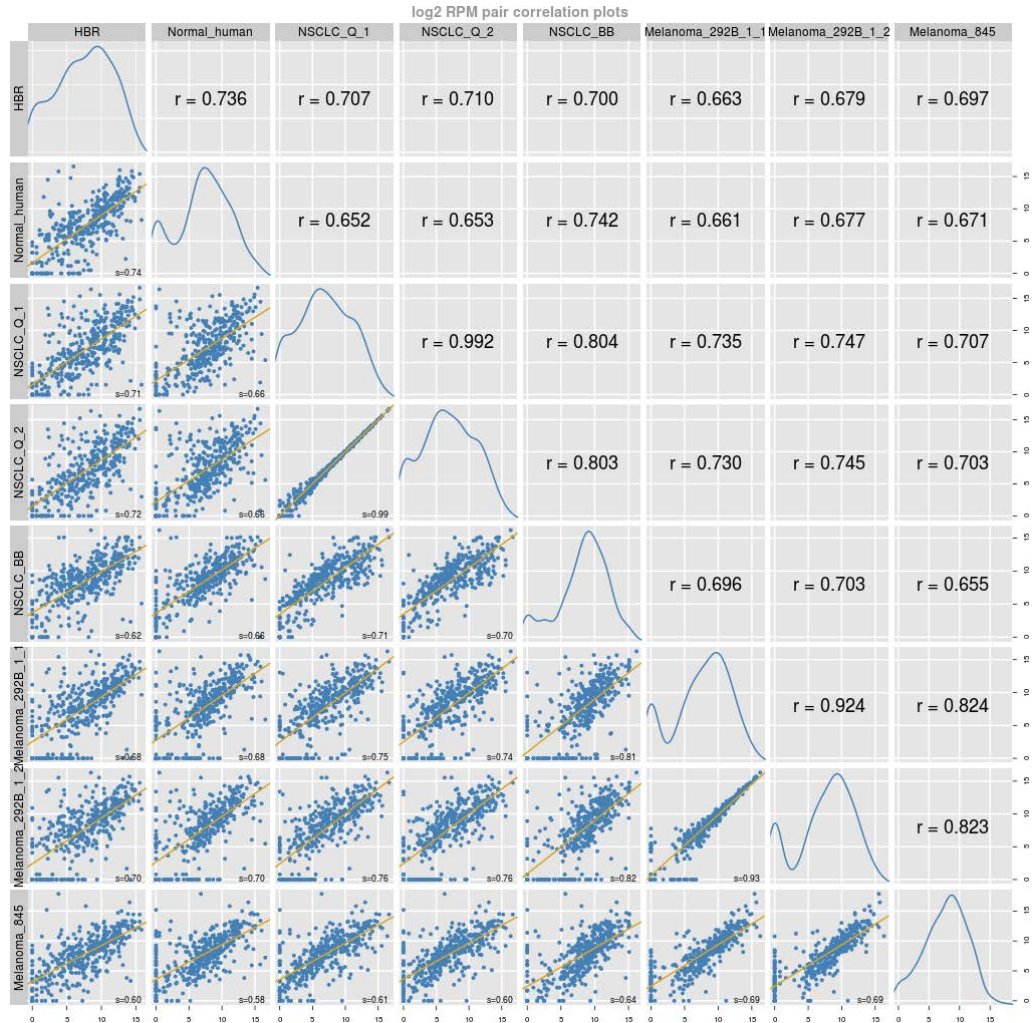
PCA plot for samples



Principal component analysis (PCA) using top 50 genes with the highest variance to assess relationship between barcoded samples.

Sample
Correlation tab

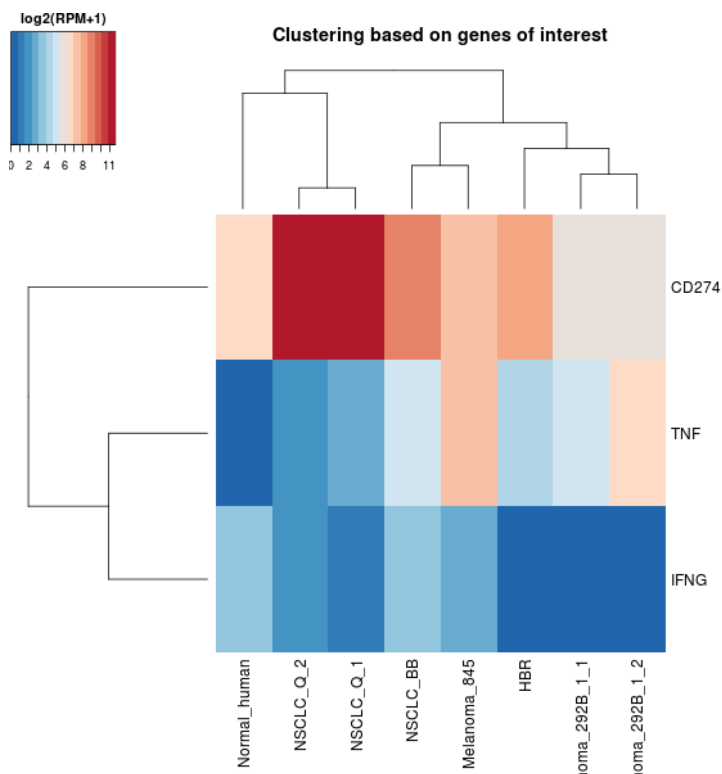
Barcode read pair correlation plot



Lower panels show (log2 RPM+1, RPM: read per million of mapped reads) values plotted for each pair of barcodes, with linear least squares regression line overlaid and line slope reported. Upper panels show Pearson correlation r-values for the regression line. Diagonal panels show the frequency density plot for the individual (log RPM+1) values for each barcode. (If only one barcode has reads, a density plot is displayed.) Click the plot to open an expanded view.

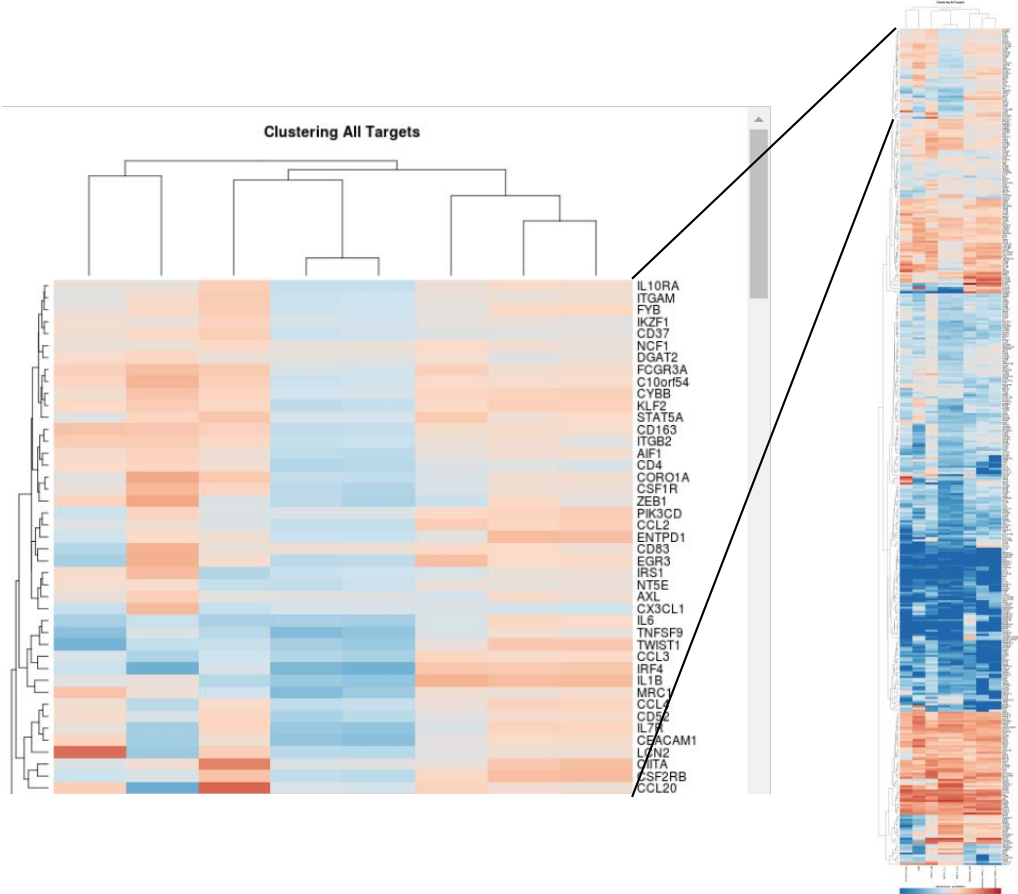
Analysis of Selected Targets

If an optional .bed file specifying a subset of target "Genes of interest" is included in the analysis, a heatmap is produced with clustering based only on the expression of these genes.



Analysis of All Targets tab

This analysis displays a heatmap of all targets using normalized read count per million (RPM) of mapped reads.



Downloadable reports

The following reports are available for download as tab-delimited text files, compatible with Microsoft™ Excel™ or similar applications.

At the bottom of the screen are links for downloading raw analysis output files:

Report hyperlink	Description
Download Barcode Summary Report	A table listing each barcode's sample name, total reads, aligned reads on targets, and number of targets detected.
Download absolute read counts data	A table listing read counts for each barcoded sample along with gene annotations.
Download RPM data (normalized by total read counts)	A table listing RPM (Read count Per Million mapped reads) for each barcoded sample along with gene annotations. RPM is calculated as: (read count) × 10 ⁶ / total number of mapped reads
Download mean housekeeping scaled log2 RPM data	A table listing housekeeping-gene normalized, log2-transformed read counts for each barcoded samples along with gene annotations. Conceptually, these values are read count normalized by the average expression of housekeeping (<i>hk</i>) genes rather than by the total number of mapped reads as described above. The values are calculated as: $\log_2(\text{count} + 1) - \frac{\sum(\log_2(\text{hk counts} + 1))}{\text{number of } hk \text{ gene}} + \log_2(10^6)$ <p>These values are useful for differential analysis when a large proportion of the target genes (non-housekeeping genes) are expected to be differentially expressed or when the expression levels of the housekeeping genes in the 2 groups differ significantly.</p>
Download CHP files normalized by RPM	The RPM data is converted to CHP file format for use with Affymetrix™ Transcriptome Analysis Console (TAC) software. The downloaded .ZIP file contains all the CHP files from the sequencing run. Each barcoded sample has 1 CHP file.
Download CHP files normalized by mean housekeeping genes	Similar to the above CHP file, but data in these CHP files are normalized by housekeeping genes.
Download background expression from genomic DNA and H ₂ O neg_control	A table containing background expression (in absolute read count) from 4 experiments using genomic DNA and H ₂ O as negative control samples.

Affymetrix™ Transcriptome Analysis Console (TAC) analysis

System requirements

Transcriptome Analysis Console Software (64-bit)	
Operating system ^[1]	<ul style="list-style-type: none"> Windows™ 7 professional (64-bit) Windows™ 10 professional (64-bit)
Processor	2.83 GHz Intel™ Pentium® Quad Core
Memory (RAM)	8 GB (minimum) 16 GB (recommended)
Disk space	150 GB HD + data storage
Web browser	Internet Explorer® 8.0 and above

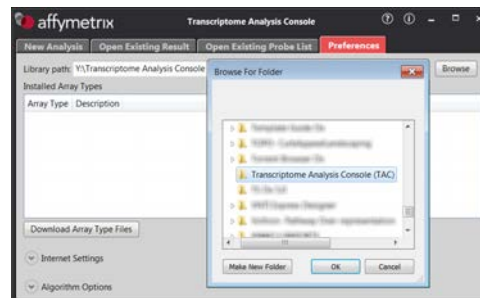
^[1] TAC software is not compatible for use with Mac OS™. To use TAC on Mac OS™, a virtual machine with Windows™ 64-bit installation is required on the Mac OS™ host system.

Download and install TAC software

Affymetrix™ Transcriptome Analysis Console (TAC) software is available for download from the Affymetrix website.

- Click or copy/paste the following URL into your browser: **Affymetrix/TAC**.
Note: You will need to log in to download the software. If you do not have an existing account, please create a new account.
- Click **Download**.
- Extract the downloaded .zip file, double-click **TAC3.1_setup.exe**, then click **Run** and follow the prompts to install the program.
Note: You must install the .NET framework if it is not already installed.
- Download TAC Immune Response annotation files from the OncoMine™ Immune Response Research Assay product page.
 - In the **thermofisher.com** home page search using the OncoMine™ Immune Response Research Assay catalog number (Cat. No. A32881) or (Cat. No. A32928).
 - In the OncoMine™ Immune Response Research Assay product page, click **Download annotation files**.
A .zip file containing the TAC Immune Response annotation files will automatically begin to download.
 - Extract the .zip file to the location of your choice.
- Double-click the **Transcriptome Analysis Console** icon to start the software. You will be asked to define a library path.

6. Select or create a new annotation library folder where annotation files will be stored. To define the library path at any time:
 - a. Click the **Preferences** tab, then click **Browse**.
 - b. Navigate to and select your annotation folder, then click **OK**.



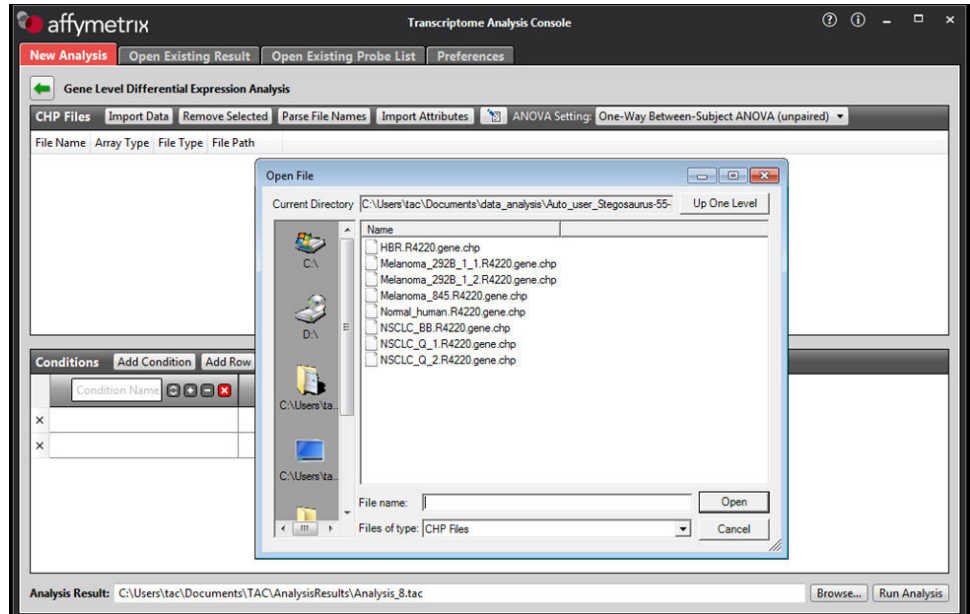
7. Copy the following two annotation files from step 4 into your annotation library folder:
 - Immune-response.tac_analysis_configuration
 - Immune-response_v3.transcript.csv
8. In the **Preferences** tab, set **Array Type** to "Immune-response" and **Description** to "Gene level differential expression analysis".
9. Click **Algorithm Options**, then select **Immune-response_v3.transcript.csv** in the **Gene** dropdown list.



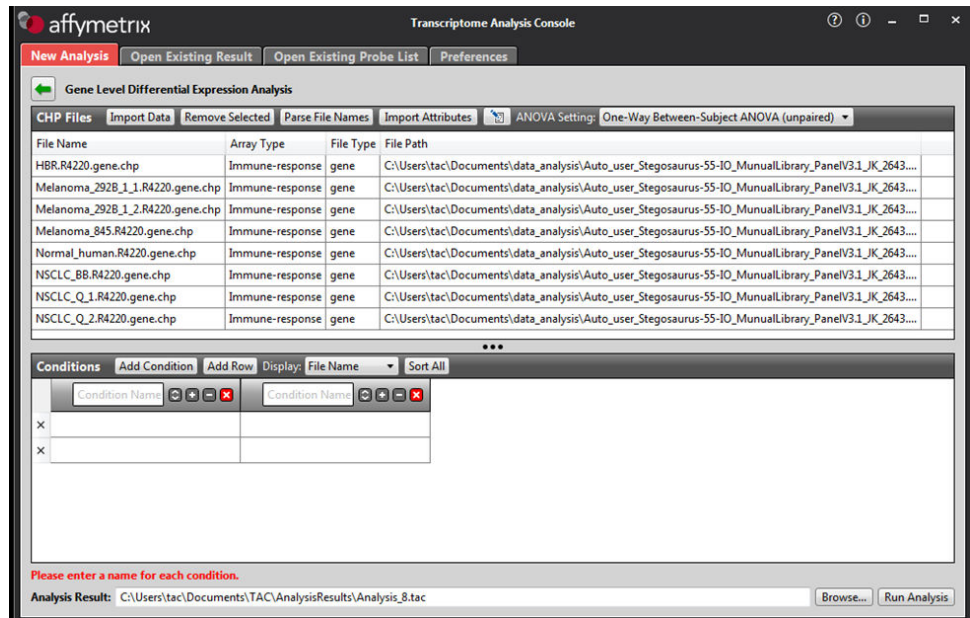
Perform TAC expression analysis

1. Download both CHP .zip files from the Torrent Suite™ software (see "Downloadable reports" on page 40 for more information).
2. Extract the .zip files, then save the .chp files (one .chp file per sample) in your annotation library folder.
3. Start the TAC software, click the **New Analysis** tab, then in the **Select Analysis** window click **Gene Level Differential Expression Analysis**.

4. Click **Import Data**, then navigate to and select the downloaded .chp files, then click **Open**.

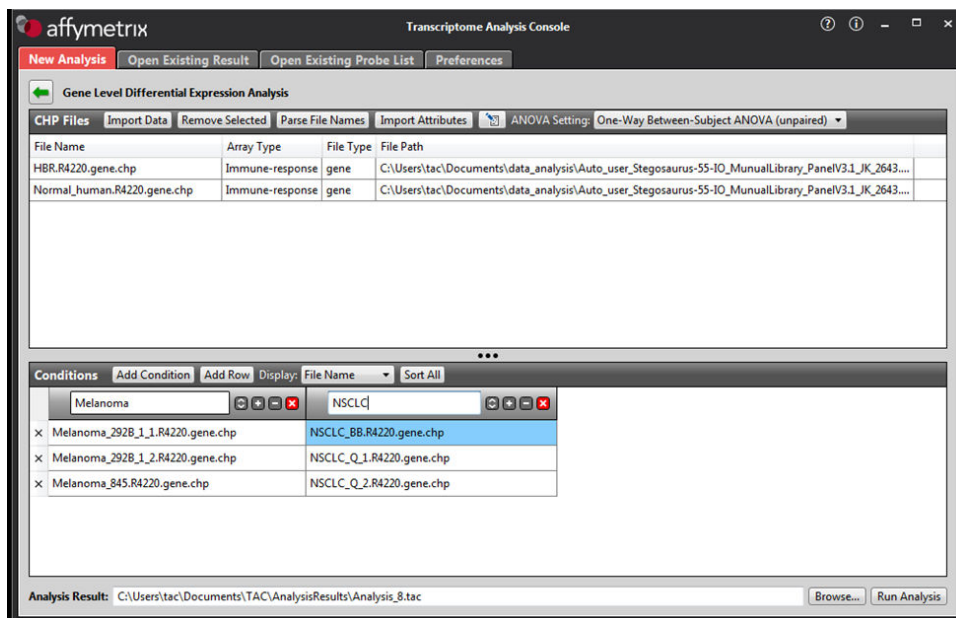


The data files are imported into the TAC and available for analysis.




5. Group the samples for differential analysis.
 - a. Enter a name for each condition in the **Condition Name** fields.
 - b. Select and drag each sample to be included in the analysis into one of the two **Conditions** columns in the lower pane.

- c. Click **Add Condition** or **Add Row** to expand the analysis.



6. Click **Browse**, then navigate to and select the location to save the analysis results, then click **Open**.
7. Click **Run Analysis**.

Download (click ) the *Transcriptome Analysis Console (TAC) 3.1 User Guide* (P/N 703150) for details on the analysis.



Tips and troubleshooting

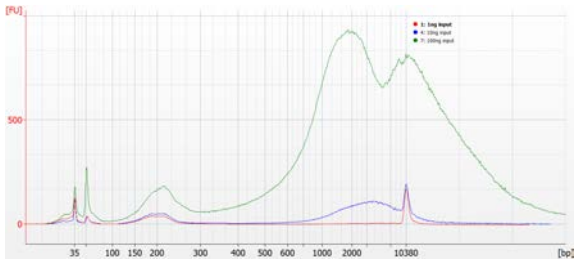
Tips

- 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™ Clear Adhesive Film Kit. 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™ adapters to avoid handling and diluting adapters. Alternatively, combine and dilute Ion Xpress™ adapters in large batches, then carefully aliquot into 96-well plates.
- When preparing libraries for the first time verify sample RNA integrity by performing the functional RNA quantification, see page 49 for more information.
- If you are performing qPCR quantification, library amplification is unnecessary and the tube of Platinum™ PCR SuperMix High Fidelity supplied in the kit can be used for other applications. See the *Platinum™ PCR SuperMix High Fidelity User Guide* for instructions.
- When transfer to a new plate is specified, solutions can be transferred to a clean well in the same plate instead, if desired.
- If library yield is below 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” 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.
- During target amplification, 98°C can be used for the enzyme activation and denaturation steps for some panels, especially those targeting cDNA. This change can improve underperforming amplicons.

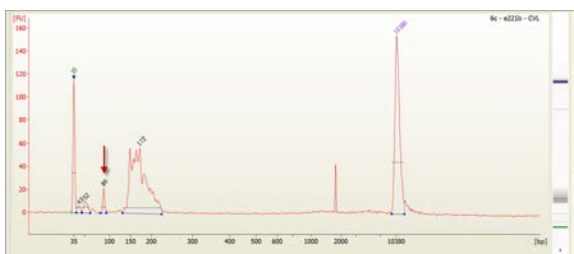
Troubleshooting

Library yield and quantification

Observation	Possible cause	Recommended action
Library concentration is low—general (Library concentration is NOT indicative of quality.)	Sample DNA or RNA was mis-quantified.	Requantify sample DNA using the TaqMan® RNase P Detection Reagents Kit; quantify RNA with Qubit™ 2.0 or 3.0 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.	
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 Qubit™ Fluorometer.
	More than 100 ng of sample DNA/RNA was used.	Add less DNA/RNA, or decrease target amplification cycles.
Library concentration is high as measured on the Agilent™ 2100 Bioanalyzer™ instrument	Markers are mis-assigned.	Ensure that markers are assigned correctly.

Observation	Possible cause	Recommended action
<p>High molecular weight material is present on the Agilent™ 2100 Bioanalyzer™ instrument or library concentration is high on the Qubit™ Fluorometer</p>  <p>Example Agilent™ 2100 Bioanalyzer™ analysis showing presence of high molecular weight material.</p>	<p>High molecular weight DNA was not removed during purification of the amplified library (does not interfere with sequencing).</p>	<p>Remove less supernatant in the first-round (0.5X) purification and be sure not to disturb bead pellet.</p> <p>Increase AMPure™ XP Reagent volume from 25 µL (0.5X) to 35 µL (0.7X) in the first-round purification.</p>
	<p>Inserts are concatamerizing during the ligation step.</p>	<p>Reduce nucleic acid input amount.</p>
		<p>Requantify sample(s) with Qubit™ 2.0 or Qubit™ 3.0 Fluorometer.</p>
		<p>Reduce target amplification cycle number.</p>

Other

Observation	Possible cause	Recommended action
<p>Adapter dimers are present on the Agilent™ 2100 Bioanalyzer™ instrument at 90–105 bp or Adapter dimers are present during sequencing</p>  <p>Adapter dimers. Barcode adapters run at ≈53 bp, and barcode adapter dimers run at ≈105 bp.</p>	<p>Purification was inefficient.</p>	<p>In unamplified library purification, decrease AMPure™ XP Reagent volume from 45 µL (1.5X) to 30 µL (1X).</p> <p>In amplified library purification, decrease AMPure™ XP Reagent volume in second round from 60 µL (1.2X) to 50 µL (1.0X).</p>
	<p>Adapter dimers formed during reaction setup or during digestion.</p>	<p>Do not combine Adapters, DNA Ligase, and Switch Solution prior to addition.</p> <p>Use a 65°C temperature incubation instead of 60°C during the amplicon digestion step.</p>
	<p>Adapter concentration was too high.</p>	<p>Ensure that barcode adapters are diluted properly.</p>
<p>The number of on-target reads is lower than expected</p>	<p>Unknown.</p>	<p>Increase the number of target amplification cycles by 2.</p>
	<p>Sample ID Panel targets were counted as off-target reads.</p>	<p>Add back the on-target reads from the Sample ID Panel.</p>
<p>Barcode representation is uneven (Equalizer™ kit not used)</p>	<p>Library quantification was inaccurate.</p>	<p>Use the Ion Library TaqMan® Quantitation Kit for the most specific and accurate library quantification.</p>
	<p>Library combination was inaccurate.</p>	<p>Dilute libraries to 50 pM, then combine equal volumes.</p>

Observation	Possible cause	Recommended action
Percentage of polyclonal ISPs is high (>40%)	Library input was too high.	Decrease amount of library added to the template preparation reaction by 50%.
	Library was mis-quantified.	Ensure that library was quantified accurately.
	Other.	Check the appropriate template preparation user guide for more information.
Low quality ISPs are present at high percentage (> 15%)	Library input was too low.	Double the volume of library used in template preparation.
		Use a fresh dilution of library prepared in a low-bind tube.
	Other.	Check the appropriate template preparation user guide for more information.



Supplemental information

Functional RNA quantification

Determine the concentration of your RNA samples by quantitative RT-PCR in triplicate.

IMPORTANT! Use non-stick RNase-Free Microfuge Tubes when preparing samples and RNA standards.

1. Prepare serial dilutions of the RNA standard (HL60 total RNA) as indicated in the following table. Label each tube with the concentration, then use these concentrations in the qPCR instrument software. Keep RNA standard dilutions on ice until use.

Tube #	Dilution concentration (ng/ μ L)	Volume dilution	Nuclease-Free Water	Total volume
—	100 ng/ μ L	2 μ L stock HL60 total RNA	18 μ L	20 μ L
1	50 ng/ μ L	10 μ L	10 μ L	20 μ L
2	12.5 ng/ μ L	5 μ L	15 μ L	20 μ L
3	3.13 ng/ μ L	5 μ L	15 μ L	20 μ L
4	0.78 ng/ μ L	5 μ L	15 μ L	20 μ L
5	0.2 ng/ μ L	5 μ L	15 μ L	20 μ L
6	0.05 ng/ μ L	5 μ L	15 μ L	20 μ L
NTC	—	—	20 μ L	20 μ L

2. Calculate, then prepare sufficient reaction mix for triplicate reactions of each sample, standard, and NTC as indicated in the following table. An overage is included to accommodate pipetting.

Component	Volume/reaction	32 reactions ^[1]
4X TaqMan [®] Fast Virus 1-Step Master Mix	8.75 μ L	315 μ L
20X TaqMan [®] Gene Expression Assay, GUSB	1.75 μ L	63 μ L
Nuclease-Free Water	21 μ L	756 μ L
Total volume	31.5 μ L	1134 μ L

^[1] Up to 32 reactions (25 samples + 6 standards + 1 NTC) can be analyzed in a single 96-well plate.

3. Close the tube, vortex to mix, then briefly centrifuge to collect the contents. Keep on ice until use.

9. Define the experiment properties in your qPCR instrument software. For the QuantStudio™ Software use:
 - 96-well (0.2 mL reaction volume)
 - Block type—Standard
 - Run mode—Standard
 - Experiment type—Standard curve, enter the concentrations of the RNA standards
 - Chemistry—TaqMan® reagents
 - Assay reporter—FAM™ dye
 - Quencher—NFQ-MGB
 - Passive reference—ROX™ dye

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

Stage	Temperature	Time
Hold	50°C	20 minutes
Hold	95°C	2 minutes
40 cycles	95°C	3 seconds
	60°C	60 seconds

11. When cycling is complete, set the threshold value to 0.2, then click **Analyze**. The parameters of the line of best fit for the standard curve should be:

Parameter	Range
Slope	-3.0 to -3.6
R-squared value	0.9-1.0
Efficiency	90-110%

The results are now ready for export. Use the average of the triplicate sample reactions as the functional RNA quantity.

Note: If samples were diluted prior to analysis, multiply the average by the dilution factor to obtain the functional RNA quantity.

Install preloaded Reference Library file

Before you can set up an Oncomine™ Immune Response Research Assay Run Plan, the preloaded Reference Library file must be imported and installed in the Torrent Suite™ Software.

1. Log in to the Torrent Server where you want to install the reference files.
2. Click the ⚙️ "Gear" tab, then select **References** from the dropdown list.
3. Click **Import Preloaded Ion References**, find **Immune Response (v3.1)** in the list, then click the adjacent **Import** button.

The Reference Library file "**ImmuneResponse_v3.1**" automatically starts downloading and appears in the Reference Sequences list.

Short Name	Description	Notes	Date	Index version	Status	Enabled
ImmuneResponse_v3.1	Immune Response (v3.1)		2016/10/28 12:13 PM	tmap-43	complete	true
GRCh38.p2.mask1	GRCh38.p2.mask1		2016/10/24 03:51 PM	tmap-43	indexing	false

In the Reference Sequences list you can follow the process: **downloading** ▶ **preprocessing** ▶ **complete**. When complete the Reference Library file is available in the Reference Library dropdown list during Run Plan creation.

Install Target Regions .bed files

Contact your field service representative to obtain the latest version of the Oncomine™ Immune Response Research Assay Target Regions .bed file.

1. Extract the Target Regions .zip file to the location of your choice.
2. Log in to the Torrent Server where you want to install the reference files.
3. Click the ⚙️ "Gear" tab, then select **References** from the dropdown list.
4. Upload the Target Region .bed file:
 - a. In the left navigation menu, click **Target Regions**, then click the **Add Target Regions** button.
 - b. Select **ImmuneResponse_v3.1** from the **Reference** dropdown list.

- c. Click **Select File**, then navigate to and select the Immune Response Target Regions .bed file.

New Target Regions

Target Regions File :
Please select a BED file to upload.

Reference : ImmuneResponse_v3.1 - Immune Response (v3.1)

Description : optional

Notes : optional

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

Note: .bed files specifying a subset of target "Genes of interest" for sample clustering can be uploaded now if desired.

The **Target Regions** .bed file automatically starts downloading to your Torrent Server. When complete the **Target Regions** .bed file is available in the **Target Regions** dropdown list during Run Plan creation.

Manually launch the immuneResponseRNA plugin

1. In the Torrent Browser, click **Data ▶ Completed Runs & Reports** tab, then click the **Report Name**. The run report opens.
2. In the run report page, scroll down to the **Plugin Summary** tab. Click **Select Plugins to Run** to open the **Select a plugin** dialog.
3. Click **immuneResponseRNA** to launch the plugin.
4. Select **AmpliSeqRNA** from the **Library Type** dropdown list.
5. Select **ImmuneResponse_v3.1_target_designed_20160908.bed** from the **Target Regions** dropdown list.
6. (Optional) Select your target gene subset .bed file in the **Genes of interest** dropdown list.

ImmuneResponseRNA Plugin

Reference Genome: ImmuneResponse_v3.1_20160908

Library Type:

Targeted Regions:

Genes of interests:

Add new gene list:

Filter Barcodes:

Note: We recommend that this .bed file is created by deleting unwanted targets from the complete Target Regions .bed file so that the format and amplicon names are consistent.

7. (Optional) Upload a **Genes of interest** .bed file.

Note: Target gene subset .bed files can also be uploaded directly to your Torrent Suite™ software, see “Install Target Regions .bed files” on page 52 for more information.

- a. Click **Add genes of interest...**
 - b. Select **ImmuneResponse_v3.1_reference_20160908** from the **Reference** dropdown list.
 - c. Click **Select File**, then navigate to and select the appropriate Target Regions .bed file.
 - d. Click **Open**, then click **Upload Target Regions File**.
 - e. Close the **ImmuneResponseRNA Plugin** dialog, then repeat steps 2–6.
8. Click **Submit** to launch the analysis.

Note: We recommend leaving **Filter Barcodes** deselected (default). It is designed to remove barcodes with few reads from the report, but could prevent non-template control (NTC) samples from being reported.



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, etc). To obtain SDSs, see the “Documentation and Support” section in this document.
-

Chemical safety



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

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

Biological hazard safety



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

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
www.cdc.gov/biosafety/publications/bmb15/BMBL.pdf
 - World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf
-

Documentation and support

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 in services and support, including:

- Worldwide contact telephone numbers
- Product support, including:
 - Product FAQs
 - Software, patches, and updates
 - Training for many applications and instruments
- Order and web support
- Product documentation, including:
 - 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 found on Life Technologies' website 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.

