iontorrent

Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay

USER GUIDE

for use with:
Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay – GS Manual
Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay – GS Chef Ready
Ion Chef™ System
Ion GeneStudio™ S5 Series System

Catalog Numbers A51305 and A51306
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Revision C.0

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

Revision history: MAN0024915 C.0 (English)

Revision	Date	Description
C.0	16 December 2023	• Reorganized chapter 1 into 2 chapters. • Updated Ion AmpliSeq™ Chef Supplies DL8 (Part No. A29027) to include the PCR Plate Frame component. • Updated instructions to include loading the PCR Plate Frame. See "Prepare libraries on the Ion Chef™ Instrument" on page 44. • Removed information about discontinued TaqMan™ 2019-nCoV Assay Kit v1 (A47532). • Removed information about discontinued TaqPath™ COVID-19 CE-IVD RT-PCR Kit (A51738). • Protocol updates.
B.0	11 June 2021	• Corrected cycling conditions for the Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay – GS Chef Ready • Corrected product names. • Corrected Ct conversion table for TaqPath™ kits. • Corrected cycling conditions for manual library preparation. • Added purification step after library amplification during quantification or normalization.
A.0	28 May 2021	New document for the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel.

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

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Contents

1 Product information

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

Product description

The Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel is a comprehensive next-generation sequencing (NGS) assay to enable research and surveillance of the SARS-CoV-2 virus, which causes COVID-19, through complete genome sequencing. The panel design includes the addition of new variant-tolerant primers to broaden and improve the coverage for variants of concern and increase the sensitivity of the panel to enable detection from lower viral titer samples.

Two versions of the panel are available.

- Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay – GS Manual (Cat. No. A51305) for manual library preparation
- Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay – GS Chef Ready (Cat. No A51306) for automated library preparation on the Ion Torrent™ Ion Chef™ System.

The kit includes the following key features.

- Provides >99% coverage of the SARS-CoV-2 viral genome and variants, including all known serotypes.
- Complete genome sequencing from as low as 50 viral copies and obtain virus sequence from as low as 5 viral copies.

Software compatibility

The Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel is compatible with Torrent Suite™ Software 5.12 and later. We recommend updating your Torrent Server, Ion Chef™, and Ion sequencer instrument software to the latest available versions before using this kit.

Contents and Storage

Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay – GS Manual

Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay – GS Manual (Cat. No. A51305) is used to prepare barcoded sample libraries from RNA. The Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel and the Ion AmpliSeq™ Library Kit Plus are provided.

The Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel consists of two 5X primer pools that target 237 amplicons specific to the SARS-CoV-2 (the virus that causes COVID-19) and 5 human controls. With an amplicon length range of 125–275 bp, the panel provides >99% coverage of the SARS-CoV-2 genome (~30 kb) and covers all known serotypes.

Contents	Amount	Storage
Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel		
5X SARS-CoV-2 Insight Panel - Pool 1 (green cap)	96 reactions	–30°C to –10°C
5X SARS-CoV-2 Insight Panel - Pool 2 (grey cap)		
Ion AmpliSeq™ Library Kit Plus (Cat. No. A35907, 96 reactions)[1]		
5X Ion AmpliSeq™ HiFi Mix (red cap)	480 µL	–30°C to –10°C
FuPa Reagent (brown cap)	192 µL	
Switch Solution (yellow cap)	384 µL	
DNA Ligase (blue cap)	192 µL	
25X Library Amp Primers (pink cap)	192 µL	
1X Library Amp Mix (black cap)	4 × 1.2 mL	
Low TE	2 × 6 mL	15°C to 30°C Can be stored at –30°C to –10°C for convenience.

[1] Also available separately.

Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay – GS Chef Ready

The Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay – GS Chef Ready (Cat. No. A51306) is used to prepare barcoded sample libraries from RNA. The kit consists of the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel and the Ion AmpliSeq™ Kit for Chef DL8.

The Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel consists of two 2X primer pools that target 237 amplicons specific to the SARS-CoV-2 (the virus that causes COVID-19) and 5 human controls. With an amplicon length range of 125–275 bp, the panel provides >99% coverage of the SARS-CoV-2 genome (~30 kb) and covers all known serotypes.

Contents	Amount	Storage
Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay – GS Chef Ready		
2X SARS-CoV-2 Insight Panel - Pool 1	32 reactions	–30°C to –10°C
2X SARS-CoV-2 Insight Panel - Pool 2		
Ion AmpliSeq™ Kit for Chef DL8 (Cat. No. A29024, 32 reactions) [1]		
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 Cartridges are shipped at ambient temperature but must be stored at 2°C to 8°C on arrival.
Ion AmpliSeq™ Chef Supplies DL8 (per insert) (Part No. A29027) • Ion AmpliSeq™ Tip Cartridge L8 • PCR Plate Frame • PCR Frame Seal • Enrichment Cartridge	1 box with 4 inserts	15°C to 30°C
IonCode™ 0101–0132 in 96 Well PCR Plates (dried) (Part No. A29028) Set includes 4 PCR plates. • IonCode™ 0101–0108 in 96 Well PCR Plate (red cap) • IonCode™ 0109–0116 in 96 Well PCR Plate (yellow cap) • IonCode™ 0117–0124 in 96 Well PCR Plate (green cap) • IonCode™ 0125–0132 in 96 Well PCR Plate (blue cap)	1 set of 4 plates	15°C to 30°C

[1] Also available separately.

Workflow—Manual library preparation

Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel manual library preparation workflow

Reverse transcribe RNA with the Ion Torrent™ NGS Reverse Transcription Kit (page 24)

Amplify the cDNA targets (page 26)

Partially digest the DNA and cDNA amplicons (page 27)

Ligate adapters to the amplicons and purify (page 27)

Quantify the library by qPCR (page 30) **or Equalize the library** (page 34)

Quantify or normalize the libraries using one of these options.

- Amplify the libraries, then quantify libraries by qPCR.
- Amplify the libraries, then normalize libraries using the Ion Library Equalizer™ Kit.
- Alternately, libraries can be quantified by Qubit™ Fluorometer. See “Quantify the amplified library with a Qubit™ Fluorometer” on page 66.

Workflow—Automated library preparation using the Ion Chef™ System

Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel automated library preparation workflow

Create a Sample Set (page 40)

You can use the Sample Set to create a Planned Run. The information from the Sample Set and individual samples within the Sample Set are prepopulated in the Planned Run workflow bar steps and Planned Run template.

Reverse transcribe RNA with the Ion Torrent™ NGS Reverse Transcription Kit (page 42)

RNA is added to the barcode plate and reverse transcribed to generate cDNA for library preparation.

Prepare libraries on the Ion Chef™ Instrument (page 44)

- Load the barcode plate with cDNA samples on the Ion Chef™ Instrument for library preparation.
- Install reagents and consumables on the Ion Chef™ Instrument.
- Enter panel specific amplification parameters, perform automated library preparation and pooling of barcoded sample libraries on the Ion Chef™ Instrument.

2 Reagents, supplies, and required materials

Required materials not supplied

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

Item	Source
One of the following barcode adapter kits. Ion Torrent™ IonCode™ Barcode Adapters 1–384 Kit Ion Torrent™ Ion Xpress™ Barcode Adapters Kit	 A29751 4474517[1]
(For automated library preparation only) Ion Torrent™ Ion Chef™ System	4484177
One of the following Applied Biosystems™ real-time PCR instruments. • 7500 Real-Time PCR System • 7900HT Fast Real-Time PCR System[2] • StepOne™ Real-Time PCR System • StepOnePlus™ Real-Time PCR System • ViiA™ 7 Real-Time PCR System • QuantStudio™ 3 Real-Time PCR System • QuantStudio™ 5 Real-Time PCR System • QuantStudio™ 7 Flex Real-Time PCR System • QuantStudio™ 12K Flex Real–Time PCR System	Various
Agencourt™ AMPure™ XP Reagent	NC9959336, NC9933872 **fisherscientific.com**
Ion Torrent™ NGS Reverse Transcription Kit[3]	A45003

(continued)

Item	Source
One of the following Applied Biosystems™ thermal cyclers, or equivalent. • ProFlex™ 96-well PCR System • VeritiPro™ Thermal Cycler, 96-well • Veriti™ 96-Well Thermal Cycler[2] • 2720 Thermal Cycler[2] • GeneAmp™ PCR System 9700 96-Well[2] or GeneAmp™ PCR System 9700 Dual 96-Well[2]	Various
Ion Torrent™ Ion Library TaqMan™ Quantitation Kit	4468802
Applied Biosystems™ MicroAmp™ Optical 96-Well Reaction Plate or Applied Biosystems™ MicroAmp™ Optical 96-Well Reaction Plate with Barcode	N8010560 or 4306737
Applied Biosystems™ MicroAmp™ Fast Optical 96-Well Reaction Plate	4346907
Applied Biosystems™ MicroAmp™ Clear Adhesive Film	4306311
Applied Biosystems™ MicroAmp™ Optical Film Compression Pad	4312639
Invitrogen™ DynaMag™–96 Side Magnet or other plate magnet	12331D
Eppendorf™ DNA LoBind™ Microcentrifuge Tubes, 1.5 mL	13-698-791 **fisherscientific.com**
Invitrogen™ Nuclease-Free Water (not DEPC-Treated)	AM9932
Ethanol, Absolute, Molecular Biology Grade	BP2818500 **fisherscientific.com**
Pipettors, 2–200 µL, and low-retention filtered pipette tips	**MLS**
(Optional) Ion Torrent™ Ion Library Equalizer™ Kit	4482298

[1] Various kits are available. For more information, see **thermofisher.com**.

[2] Supported but no longer available for purchase.

[3] If you are using another reverse transcription system, such as the Invitrogen™ SuperScript™ VILO™ cDNA Synthesis Kit, contact support.

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	
Applied Biosystems™ real-time PCR instruments (see “Required materials not supplied” on page 12)	Various
96-well plate centrifuge	**MLS**
Invitrogen™ Qubit™ 4 Fluorometer[1]	Q33238
Recommended for nucleic acid isolation	
Applied Biosystems™ MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit	A42352 or A48310
Recommended for nucleic acid quantification	
Invitrogen™ Qubit™ dsDNA HS Assay Kit (DNA)	Q32851, Q32854,
Invitrogen™ Qubit™ RNA HS Assay Kit (RNA)	Q32852, Q32855
Applied Biosystems™ TaqMan™ RNase P Detection Reagents Kit	4316831
Recommended for SARS-CoV-2 quantification	
TaqPath™ COVID-19 Combo Kit	A47814

[1] Invitrogen™ Qubit™ 2.0 Fluorometer or later are supported.

3 Before you begin

Before first use—Install plugins and Run Plan templates

IMPORTANT! If the computer running Torrent Suite™ Software is not connected to the network, you must manually upload the SARS-CoV-2 plugins and Run Plan templates. Contact support for assistance.

You must download or import the following objects.

- Reference file ("Import the reference sequence file")
- Plugins ("Download plugin files" on page 16)
- Run Plan files and SARS_CoV_2_lineageID plugin ("Obtain Run Plan files and set up the SARS_CoV_2_lineageID plugin" on page 16)

Import the reference sequence file

Before you can use the reference file in Planned Runs, you must import the reference files into the software.

1. Click **(Settings)** ▸ **Reference Sequences**.
2. Click **Import Preloaded Reference Sequences**.

The **Ion References** and **Downloads** lists are displayed.

3. In the **Ion References** list, find **Ion_AmpliSeq_SARS-CoV-2-Insight_Reference**, then click **Import**.
   The file import status appears in the row of the `Ion_AmpliSeq_SARS-CoV-2-Insight_Reference` file in the **Ion References** list.

When the status is **complete**, a compressed folder in ZIP file format is added to the **Downloads** list and the reference genome is added to the **Reference Sequences** table. You can now use the reference genome in a Planned Run.

Download plugin files

Download the following plugin files from Thermo Fisher™ Connect Platform.

1. Sign in to Thermo Fisher™ Connect Platform at **thermofisher.com/connect**.
2. In the **Applications** bar, click **(AppConnect)**.
3. In the **AppConnect** screen, in the **Resource Libraries** pane, click **Plugins**.
4. Find the following plugins in the list.

Plugin ZIP file	Plugins
`SARS_CoV_2__Insight_Research_Plugin_Package_TorrentSuiteSoftware.zip`	• generateConsensus • SARS_CoV_2_annotateSnpEff • SARS_CoV_2_coverageAnalysis • SARS_CoV_2_variantCaller
`SARS_CoV_2_lineageIDPlugin_TorrentSuiteSoftware.zip`	SARS_CoV_2_lineageID

5. Click in the row of each plugin to download the plugin to your local storage.
6. Click « to return to the **AppConnect** screen.

Obtain Run Plan files and set up the SARS_CoV_2_lineageID plugin

Contact support to obtain Run Plan templates and for assistance with setup of the SARS_CoV_2_lineageID plugin.

Run Plan ZIP file	Description
`Ion_AmpliSeq_SARS-CoV-2-Insight.20210329.results_530.zip`	Run Plan template for the Ion 530™ Chip.
`Ion_AmpliSeq_SARS-CoV-2-Insight.20210329.results_540.zip`	Run Plan template for the Ion 540™ Chip.

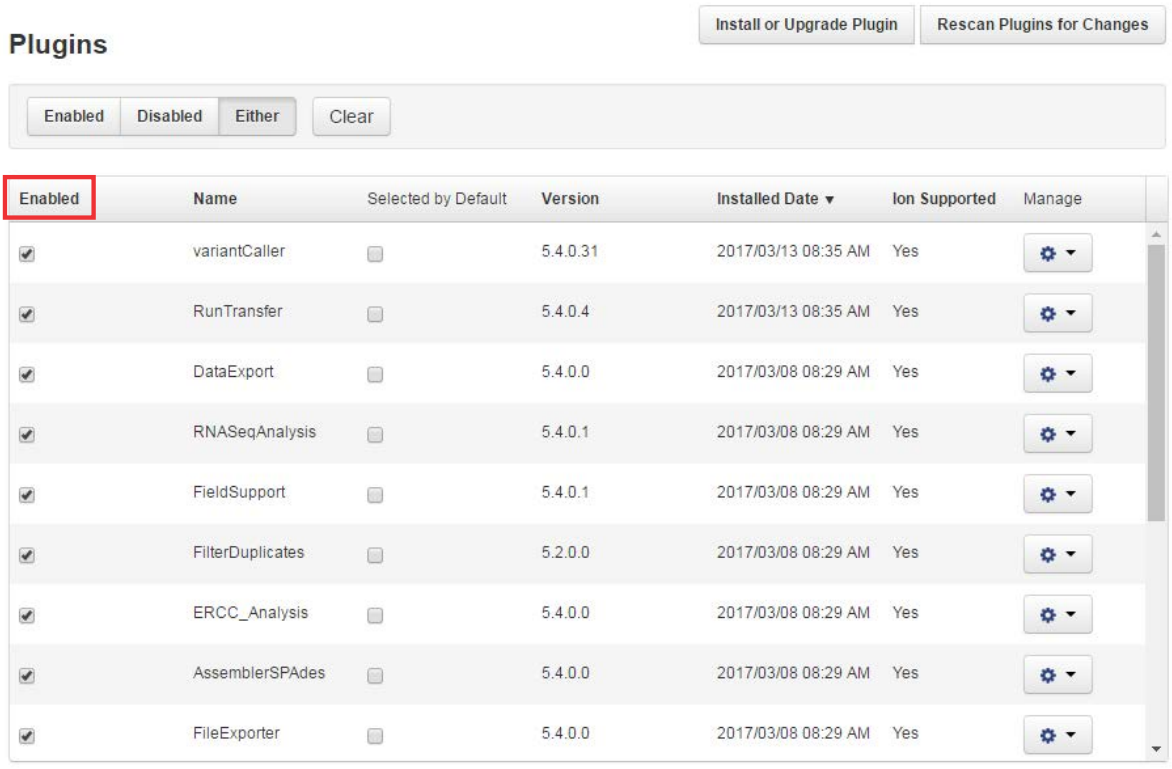
Install and enable plugins

After installing a plugin, you must enable the plugin to make the plugin available in Torrent Suite™ Software.

IMPORTANT! Do not reboot the system during the installation of plugins.

1. In Torrent Suite™ Software, click **(Settings) ▸ Plugins ▸ Install or Upgrade Plugin**.
2. Click **Select File**, browse to the location where you downloaded the plugin file, select the file, then click **Open**.

   Upload the following plugins.
   - generateConsensus
   - SARS_CoV_2_annotateSnpEff
   - SARS_CoV_2_coverageAnalysis
   - SARS_CoV_2_variantCaller
3. Contact support to install and enable the SARS_CoV_2_lineageID plugin.
4. In the **Install or Upgrade Plugin** dialog box, click **Upload and Install**.
5. Click **(Settings) ▸ Plugins**.



6. In the Plugins list, select the **Enabled** checkbox next to any installed plugin, to make it available. The plugin is immediately available.

Import analysis argument file—Online

Run Plans for the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel require the `SARS-CoV-2 Insight Research Panel Analysis Args` file.

Ensure that the server with Torrent Suite™ Software is connected to the internet. For instructions to import the file offline. See "Import analysis argument file—Offline" on page 18.

1. Click **(Settings)** ▸ **Updates**.
2. In the **Updates Products** section, find `SARS-CoV-2 Insight Research Panel Analysis Args` in the list, then click **Update**.
   - After the update of the analysis argument file is completed, **Complete** is displayed in the **Update** column.
   - During the creation of Planned Run templates, analysis argument files are uploaded to the template (see "Create a custom Planned Run template" on page 47).

Import analysis argument file—Offline

Run Plans for the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel require the `offcycle_SARS-CoV-2_Insight_analysisArgs.zip`. Use this procedure to upload the file offline.

For instructions to import the file online, see "Import analysis argument file—Online" on page 18.

1. Sign in to Thermo Fisher™ Connect Platform (**thermofisher.com/connect**).
2. In the Applications bar, click .
3. In the **AppConnect** screen, in the **Resource Libraries** pane, click **Plugins**.
4. Find `offcycle_SARS-CoV-2_Insight_analysisArgs.zip` in the list.
5. Click to download the file to local storage.
6. In Torrent Suite™ Software, click **(Settings)** ▸ **Updates** ▸ **Click to Upload and Install**.
7. In the **Install Updates** dialog box, click **Select File**, select **offcycle_SARS-CoV-2_Insight_analysisArgs.zip**, then click **Upload and Install**.
   - After the update of the analysis argument file is completed, **Complete** is displayed in the **Update** column.
   - During the creation of Planned Run templates, analysis argument files as uploaded to the template. See "Create a custom Planned Run template" on page 47.

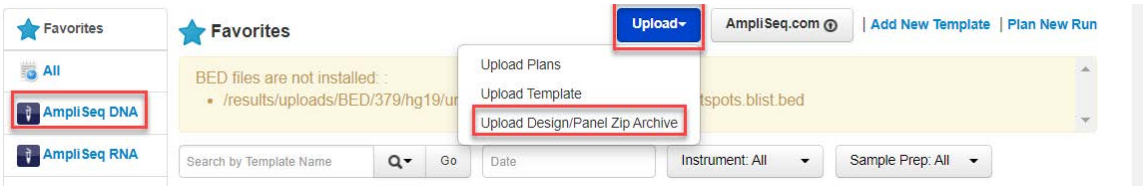
Import a Planned Run template

IMPORTANT!

- Do not restart the system during installation of planned run templates.
- If you are using Torrent Suite™ Software version 5.12, contact support to set up your Run Plan template.

1. In the **Plan** tab, in the **Templates** screen, in the research application group pane on the left, select **AmpliSeq DNA**.

2. Click **Upload** ▸ **Upload Design/Panel ZIP Archive**.



3. In the **Import Panel from Zip Archive** dialog box, click **Select File**, select the following templates, then click **Upload Panel from Zip Archive**.

   To run the Ion 550™ Chip, you must create a custom Run Plan. See “Create a custom Planned Run template” on page 47.

ZIP File	Description
`Ion_AmpliSeq_SARS-CoV-2-Insight.20210329.results_530.zip`	Run Plan template for the Ion 530™ Chip.
`Ion_AmpliSeq_SARS-CoV-2-Insight.20210329.results_540.zip`	Run Plan template for the Ion 540™ Chip.

   The template is listed in the **AmpliSeq DNA** application group. Exported Planned Run templates have “exported” appended to the front of the original template name, unless the name has been edited in the CSV file.

Before each use of the kit

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

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

Update SARS_CoV_2_lineageID plugin online

Before you begin, ensure that the server with Torrent Suite™ Software is connected to the internet.

For more information about uploading plugins to Torrent Suite™ Software, see the Torrent Suite™ Software help system.

For instructions to update the plugin offline, see “Update SARS_CoV_2_lineageID plugin offline” on page 20.

1. Click **(Settings)** ▸ **Updates**.
2. On the **Updates** screen, scroll to the **Update Plugins** section, then find the **SARS_CoV_2_lineageID** plugin.
3. In the **Update** column, **click (Settings)**, then select **Upgrade to Latest** from the dropdown list.

Update SARS_CoV_2_lineageID plugin offline

For more information about uploading plugins to Torrent Suite™ Software, see the Torrent Suite™ Software help system.

For instructions to update the plugin online, see “Update SARS_CoV_2_lineageID plugin online” on page 20.

1. Sign in to Thermo Fisher™ Connect Platform (**thermofisher.com/connect**).
2. In the **Applications** bar, click **(AppConnect)**.
3. In the **AppConnect** screen, in the **Resource Libraries** pane, click **Plugins**.
4. Find the **SARS_CoV_2_lineageID** in the list.
5. Click to download the plugin to your local storage.
6. In Torrent Suite™ Software, click **(Settings)** ▸ **Updates** ▸ **Install or Upgrade Plugin**.
7. In the **Install Updates** dialog box, click **Select File**, select **SARS_CoV_2_lineageID**, then click **Upload and Install**.
   - After the update of the analysis argument file is completed, **Complete** is displayed in the **Update** column.
   - During the creation of Planned Run templates, analysis argument files as uploaded to the template. See “Create a custom Planned Run template” on page 47.

Procedural guidelines

- Minimize freeze-thaw cycles of Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel by aliquoting into low bind tubes if 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.
- Slowly pipet viscous solutions, such as 5X Ion AmpliSeq™ HiFi Mix, FuPa Reagent, Switch Solution, DNA Ligase, Ion Torrent™ NGS 10X RT Enzyme Mix, Ion Torrent™ NGS 5X Reaction Buffer, and panels, and ensure complete mixing by vortexing or pipetting up and down several times.
- Arrange samples in alternating columns on the plate for easier pipetting with multichannel pipettes during purification with the DynaMag™–96 Side magnet.

4 Manual library preparation

If you are using an Ion 550™ Chip, contact support for assistance.

Guidelines for sample quality, viral copy number, and variant calling

- The amount of viral RNA among samples should be approximately equivalent so that the target amplification conditions you select are optimal for all samples.
- Ensure that RNA samples are quantified using TaqPath™ COVID-19 Combo Kit (Cat. No. A47814).
- A sample containing as little as 50 copies of viral RNA after isolation (25 copies per target amplification reaction) can be used to prepare an Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel library. For optimal results, we recommend a viral copy number in the 100 to 200,000 range, or an amount of *total* RNA between 1–10 ng.

Table 1 Sample quality and viral copy number

Viral copy number	Recommendations and guidelines
200 to 200,000 copies	Recommended range for optimal results.
50 to 200 copies	Only for high-quality samples without degradation. We recommend sequencing and variant detection with a minimum allele frequency of 20%. For more information about the minimum allele frequency, see the Torrent Suite™ Software help system.

- To reliably sequence low quality samples, the samples must have a viral copy number ≥200 copies per reaction. For partially degraded samples, which likely includes low titer samples, the effective copy number that can be amplified by the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel is lower than the viral copy number detected by qPCR because the qPCR products are shorter than the 250–bp fragments generated by the panel.
- Even for samples with viral titer >200 copies per reaction, you may observe reverse transcription-derived false positives if you decrease the minimum allele frequency cutoff below 0.2 (20%). Reverse transcription-related errors occur randomly across the genome. To minimize calling false-positives, be certain to amplify a sufficient number of RNA molecules and set the minimum allele frequency to at least 20%.
- See “Recommended materials” on page 14 for the recommended RNA isolation and quantification kits.

Copy number determination by qPCR

- If your qPCR data give a different relationship between C_t and copy number, this is likely a result of differences in the baseline or threshold selected. Determine the copy number of a sample according to the known copy number in control reactions.
- We recommend basing copy number on the N Protein C_t value.
- If the N Protein C_t value is not accurate, use the S Protein or ORF1ab C_t values to determine copy number.
- The copy number is only an estimate.

Table 2 Approximate copy number to C_t conversion—TaqPath™ COVID-19 RT-PCR kits

Tier	Viral copy number	TaqPath™ C_t		
		N Protein	S Protein	ORF1ab
Low	50–1,500	25–29	24–29	24–29
Medium	1,500–50,000	20–25	19–24	19–24
High	50,000–1,500,000	15–20	14–19	15–19

Reverse transcribe RNA with the Ion Torrent™ NGS Reverse Transcription Kit

To reverse transcribe using a higher volume, see “Alternate reverse transcription protocol” on page 64.

1. Combine the following components per reaction to make a master mix for the total number of reactions, plus 10% overage.

   IMPORTANT! The reverse transcription mix is viscous. Pipet solutions slowly and ensure complete mixing by vortexing or pipetting up and down several times.

Component	Volume (10–µL/rxn)
Ion Torrent™ NGS 5X Reaction Buffer	2 µL
Ion Torrent™ NGS 10X RT Enzyme Mix	1 µL
Total RNA[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 MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

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

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

STOPPING POINT Samples can be stored at 10°C for up to 16 hours in the thermal cycler. For longer term, store at –30°C to –10°C.

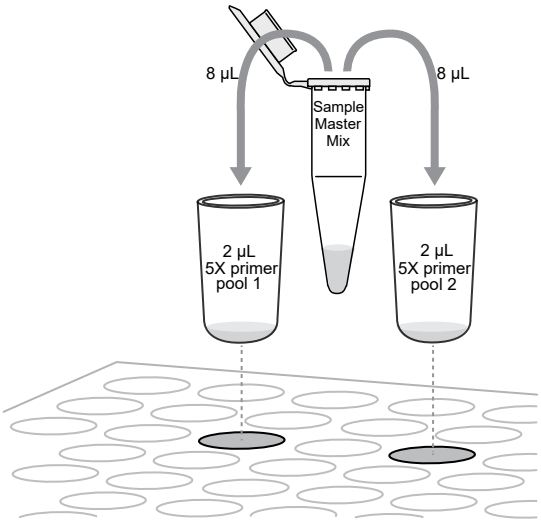
Prepare cDNA target amplification reactions

1. Set up an amplification master mix for each sample using the entire volume of the reverse transcription reaction.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	4.5 µL
cDNA	10 µL
Nuclease-free water	3.5 µL
Total volume	18 µL

2. Mix thoroughly by pipetting up and down 5 times, then transfer 8 µL of each sample-specific master mix into 2 wells of a 96-well PCR plate.

   Use a fresh pipet tip for each transfer or prewetting the pipet tip before each transfer for multiple transfers using a single tip. The amplification master mix is difficult to transfer due to viscosity.



3. Add 2 µL of primer pool 1 into the first well, and 2 µL of primer pool 2 to the second well for a total of 10 µL in each well.

4. Seal the plate with a MicroAmp™ Clear Adhesive Film, place a MicroAmp™ Optical Film Compression Pad on the plate, then place the sealed plate in a thermal cycler.

   To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

Amplify the cDNA targets

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

Run the following program to amplify the target regions.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	98°C	2 minutes
Cycle; set number according to #GUID-AA7F260F-3E74-4D1E-8E40-2A2D53641F2A/GUID-F53DD18E-66FE-4636-BDEF-B3C558AF0C80	Denature	98°C	15 seconds
	Anneal and extend	60°C	4 minutes
Hold	—	10°C	Hold

Observe the recommended cycle numbers.

Tier	Viral copy number	Number of amplification cycles
Low	50–1,500	26
Medium	1,500–50,000	20
High[1]	50,000–1,500,000	15

[1] If titers are above 1,500,000 copies, samples can be diluted.

Cycle number recommendations in the preceding table are based on qPCR quantification of viral copy number. Without qPCR quantification, use the following guidelines to determine optimal cycle number empirically.

- Low viral load suspected—26 cycles.
- High viral load suspected—20 cycles.
- Isolates or enriched viral particles—approximately 15 cycles for 2 ng input.

If you are working with samples with unknown viral load, and cannot quantify using qPCR, use 20 target amplification cycles as a starting point for manual library preparation.

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

Partially digest the DNA and cDNA amplicons

IMPORTANT! While preparing the reactions, keep each plate on ice or in a prechilled 4°C cold block.

1. Combine primer pool 1 and primer pool 2 target amplification reactions.
2. Thaw the FuPa Reagent (brown cap) on ice, gently vortex to mix, then briefly centrifuge to collect the contents.
3. Add 2 µL of FuPa Reagent to each amplified DNA or cDNA sample. The total volume per well is ~22 µL.
4. Seal each DNA or cDNA plate with a MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.

   Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
5. Place a MicroAmp™ Optical Film Compression Pad on the plate, load in the thermal cycler, then run the following program.

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

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

Ligate adapters to the amplicons and purify

When sequencing multiple libraries on a single run, you *must* ligate a different barcode to each library.

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

Ion Xpress™ Barcode Adapters require handling and dilution as described in “Combine and dilute adapters—Ion Xpress™ Barcode Adapters only”.

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

Combine and dilute adapters—Ion Xpress™ Barcode Adapters only

For each barcode X selected, prepare a mix of Ion P1 Adapter and Ion Xpress™ barcode X at a final dilution of 1:4 for each adapter. Scale volumes as necessary. Use 2 µL of this barcode adapter mix in step 3 in "Perform the ligation reaction" on page 28.

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

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

Store diluted adapters at –20°C.

Perform the ligation reaction

1. If there is visible precipitate in the Switch Solution or the tube cap after thawing, vortex or pipet up and down at room temperature to resuspend before pipetting.
2. If you have not already done so, briefly centrifuge the plate to collect the contents, then carefully remove the plate seal.
3. Add the following components in the order listed to each well containing digested amplicons. If preparing multiple non-barcoded libraries, a master mix of Switch Solution and adapters can be combined before addition.

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

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

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

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

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

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

Purify the unamplified library

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

1. Briefly centrifuge the plate to collect the contents.
2. Carefully remove the plate seal, then add 45 µL (1.5X sample volume) of Agencourt™ AMPure™ XP Reagent to each library. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.

   Visually inspect each well to ensure that the mixture is homogeneous.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.
5. Add 150 µL of freshly prepared 70% ethanol, move the plate side-to-side in the two positions of the magnet to wash the beads, then remove and discard the supernatant without disturbing the pellet.

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

   Do not overdry.

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

Quantify the libraries using one of these procedures.

- To quantify libraries using qPCR, proceed to "Quantify the library by qPCR".
- To normalize the libraries using the Ion Library Equalizer™ Kit, proceed to "Equalize the library" on page 34.
- To quantify the libraries using the Qubit™ Fluorometer, proceed to "Quantify the amplified library with a Qubit™ Fluorometer" on page 66. Optimization may be required.

During quantification or normalization, you must amplify the libraries and perform a two-step purification.

Quantify the library by qPCR

IMPORTANT! Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel libraries must be amplified before quantification to enrich amplifiable material and obtain sufficient material for accurate quantification.

Amplify the library using 1X Library Amp Mix, then purify. 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 ~100 pM, which is suitable for template preparation using an Ion template kit.

Amplify the library

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

   The 1X Library Amp Mix and 25X Library Amp Primers can be combined before addition.

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

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

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

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

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

Purify the amplified library

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

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

IMPORTANT!

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

First-round purification

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

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

Second-round purification

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

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

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

   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 briefly centrifuge to collect the contents.

   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 library by qPCR and calculate the dilution factor” on page 32.

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

Quantify library by qPCR and calculate the dilution factor

Determine the concentration of each Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel library by qPCR with the Ion Library TaqMan™ Quantitation Kit using the following steps. Analyze each sample, standard, and negative control in duplicate 20-µL reactions.

1. Prepare three 10-fold serial dilutions of the *E. coli* DH10B 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. Prepare reaction mixtures. For each sample, control, and standard, combine 20 µL of 2X Ion Library qPCR Master Mix and 2 µL of Ion Library TaqMan™ Quantitation Assay, 20X , then mix thoroughly. Dispense 11-µL aliquots into the wells of a PCR plate.

3. Add 9 µL of the diluted (1:100 to 1:1,000) Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel library or 9 µL of each control dilution to each well (two wells per sample as noted before), for a total reaction volume of 20 µL.

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

5. Program your real-time instrument.
   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.

e. The Ion Library qPCR Master Mix can be used on various instruments, as listed in the following table. The fast–cycling program was developed using the StepOnePlus™ Real-Time PCR System in Fast run mode.

IMPORTANT! When quantifying libraries made from panels with 275-bp or 375-bp designs, use standard qPCR cycling. Fast cycling can result in inaccurate quantification.

Real-time PCR System	Reaction plate	Stage	Temperature	Time
Fast run mode				
7500 Fast	96-well Fast	Hold (UDG incubation)	50°C	2 minutes
7900 HT 7900 HT Fast	96-well Fast	Hold (polymerase activation)	95°C	20 seconds
ViiA™ 7	384-well Standard	Cycle (40 cycles)	95°C	1 second
QuantStudio™ 3, 5, or 7	384-well Standard	Cycle (40 cycles)	95°C	1 second
StepOne™ StepOnePlus™	48-/96-well Fast	Cycle (40 cycles)	60°C	20 seconds
Standard run mode				
7300	96-well Standard	Hold (UDG incubation)	50°C	2 minutes
7500	96-well Standard	Hold (polymerase activation)	95°C	2 minutes
7900 HT 7900 HT Fast	96-well Standard	Cycle (40 cycles)	95°C	15 seconds
ViiA™ 7	96-well Standard	Cycle (40 cycles)	60°C	1 minute
QuantStudio™ 3, 5, or 7	96-well Standard	Cycle (40 cycles)	60°C	1 minute

6. Following qPCR, calculate the average concentration of the undiluted Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel library by multiplying the concentration that is determined with qPCR by 100 to 1,000.

7. Based on the calculated library concentration, determine the dilution that results in a concentration of ~50–100 pM.

   For example:

   - The undiluted library concentration is 300 pM.
   - The dilution factor is 300 pM/100 pM = 3.
   - Therefore, 10 µL of library mixed with 20 µL of Low TE (1:3 dilution) yields approximately 100 pM.

8. Dilute library to the appropriate concentration.

Chip	Concentration
Ion 530™ Chip	30 pM
Ion 540™ Chip	50 pM

9. Combine libraries, then proceed to template preparation, or store libraries as described below.

(Optional) Combine amplicon libraries

Multiple libraries can be combined for sequencing on the same chip.

- Ion 530™ Chip: 16–32 libraries per chip
- Ion 540™ Chip: 32–128 libraries per chip

Store libraries

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

Equalize the library

IMPORTANT!

- Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel libraries must be amplified before normalization to enrich amplifiable material and obtain sufficient material for accurate quantification.
- Standard library amplification parameters are not compatible with the Ion Library Equalizer™ Kit.

The Ion Library Equalizer™ Kit (Cat. No. 4482298) provides a method for normalizing library concentration at ~100 pM without the need for special instrumentation for quantification. First amplify the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel library, then capture the library on Equalizer™ Beads. After elution of the equalized library, proceed directly to combining libraries and/or template preparation.

Alternatively, libraries that are run on the same chip can be combined during the equalization process to improve balance and reduce hands-on time.

Ion Library Equalizer™ Kit

The Ion Library Equalizer™ Kit (Cat. No. 4482298) provides an optional, streamlined method for normalizing library concentration at 100 pM without quantification.

The kit contains reagents sufficient for 96 libraries.

Component	Amount	Storage
Equalizer™ Primers (pink cap)	200 µL	2ºC to 8ºC
Equalizer™ Capture (purple cap)	1 mL	
Equalizer™ Elution Buffer (clear cap)	10 mL	
Equalizer™ Beads (orange cap)	300 µL	
Equalizer™ Wash Buffer (clear cap)	35 mL	15–30°C[1]

[1] Can be stored at 2–8°C.

Before you begin

Warm all the reagents in the Ion Library Equalizer™ Kit to room temperature. Vortex and centrifuge all reagents before use.

Amplify the library

1. Remove the plate with purified libraries from the plate magnet, then add 50 µL of 1X Library Amp Mix (black cap) and 2 µL of Equalizer™ Primers (pink cap), to each bead pellet.

   The 1X Library Amp Mix and primers can be combined before addition.

2. Seal the plate with a MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.

   Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

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

4. Seal the plate with a new clear adhesive film, place a MicroAmp™ Optical Film Compression Pad on the plate, then load in the thermal cycler. Run the following program.

   During cycling, wash the Equalizer™ Beads, if they have not been previously washed.

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

5. (Optional) If possible after thermal cycling, briefly centrifuge the plate to collect the contents.

   The concentration of the amplified library can be confirmed by removing 2 µL of the reaction and evaluating with the Ion Library TaqMan™ Quantitation Kit. The Ion Library Equalizer™ Kit should be used only when library concentrations are consistently >4,000 pM after library amplification.

Wash the Equalizer™ Beads *(if not previously washed)*

1. Bring the Equalizer™ Beads to room temperature, then mix thoroughly.

   Beads for multiple reactions can be prepared in bulk and stored in Equalizer™ Wash Buffer at 4°C for up to 12 months until use. After 12 months, wash the beads again with an equal volume of Equalizer™ Wash Buffer.

2. For each reaction, pipet 3 µL of beads into a clean 1.5-mL tube, then add 6 µL per reaction of Equalizer™ Wash Buffer.

   For example, if you have 4 reactions, add 12 µL of beads and 24 µL of Equalizer™ Wash Buffer.

3. Place the tube in a magnetic rack for 3 minutes, or until the solution is clear.

4. Carefully remove the supernatant without disturbing the pellet, then discard.

5. Remove the tube from the magnet, add 6 µL per reaction of Equalizer™ Wash Buffer, then pipet up and down to resuspend.

Add Equalizer™ Capture to the amplified library

1. Carefully remove the seal from the plate, then add exactly 10 µL of Equalizer™ Capture reagent to each library amplification reaction.

   The final equalized library concentration depends on accurate pipetting of the Equalizer™ Capture reagent.

2. Seal the plate with a MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.

   Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

3. Incubate at room temperature for 5 minutes.

Add Equalizer™ Beads and wash

1. Gently vortex or pipet up and down to mix the washed Equalizer™ Beads.
2. If needed, carefully remove the seal from the plate, then add 6 µL of washed Equalizer™ Beads to each plate well that contains the captured library (either combined or individual).
3. Set the pipette volume to 40 µL, then pipet the mixture up and down at least 5 times to mix thoroughly.
4. Incubate at room temperature for 5 minutes.

   Check for droplets on the sides of the plate wells. If droplets are observed, seal the plate, then gently tap the plate on a hard, flat surface, or briefly centrifuge to collect the contents.
5. Place the plate in the magnet, then incubate for 2 minutes or until the solution is clear.
6. If needed, carefully remove the seal from the plate, then remove the supernatant without disturbing the pellet.

   Save the supernatant for repeat analysis, if needed.
7. Add 150 µL of Equalizer™ Wash Buffer to each reaction.
8. To wash the beads, move the plate side-to-side in the two positions of the magnet.

   If your magnet does not have two positions for shifting the beads, remove the plate from the magnet, set a pipettor to at least half the total volume, then gently pipet the contents up and down 5 times. Return the plate to the magnet and incubate for 2 minutes or until the solution clears.
9. With the plate still in the magnet, carefully remove, then discard the supernatant without disturbing the pellet.
10. Repeat the bead wash as described in step 7 through step 9.

    Ensure that as much wash buffer as possible is removed without disturbing the pellet.

Elute the Equalized library

1. Remove the plate from the magnet, then add 100 µL of Equalizer™ Elution Buffer to each pellet.
2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then 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.

   Centrifuge with enough force to collect droplets, but not pellet beads. If beads are pelleted, vortex again and centrifuge more gently.
3. Elute the library by incubating in a thermal cycler at 32°C for 5 minutes.
4. Place the plate in the magnet, then incubate at room temperature for 5 minutes or until the solution is clear.

   The supernatant contains the Equalized library at ~100 pM, which can be stored with beads for up to 1 month at 4–8°C.

5. Dilute library to the appropriate concentration.

Chip	Concentration
Ion 530™ Chip	30 pM
Ion 540™ Chip	50 pM

Proceed to template preparation or combine or store the libraries.

(Optional) Combine amplicon libraries

Multiple libraries can be combined for sequencing on the same chip.

- Ion 530™ Chip: 16–32 libraries per chip
- Ion 540™ Chip: 32–128 libraries per chip

Store libraries

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

5 Automated Library Preparation on the Ion Chef™ System

Guidelines for sample quality, viral copy number, and variant calling

- The amount of viral RNA among samples should be approximately equivalent so that the target amplification conditions you select are optimal for all samples.
- Ensure that RNA samples are quantified using TaqPath™ COVID-19 Combo Kit (Cat. No. A47814).
- A sample containing as little as 50 copies of viral RNA after isolation (25 copies per target amplification reaction) can be used to prepare an Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel library. For optimal results, we recommend a viral copy number in the 100 to 200,000 range, or an amount of *total* RNA between 1–10 ng.

Table 3 Sample quality and viral copy number

Viral copy number	Recommendations and guidelines
200 to 200,000 copies	Recommended range for optimal results.
50 to 200 copies	Only for high-quality samples without degradation. We recommend sequencing and variant detection with a minimum allele frequency of 20%. For more information about the minimum allele frequency, see the Torrent Suite™ Software help system.

- To reliably sequence low quality samples, the samples must have a viral copy number ≥200 copies per reaction. For partially degraded samples, which likely includes low titer samples, the effective copy number that can be amplified by the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel is lower than the viral copy number detected by qPCR because the qPCR products are shorter than the 250–bp fragments generated by the panel.

- Even for samples with viral titer >200 copies per reaction, you may observe reverse transcription-derived false positives if you decrease the minimum allele frequency cutoff below 0.2 (20%). Reverse transcription-related errors occur randomly across the genome. To minimize calling false-positives, be certain to amplify a sufficient number of RNA molecules and set the minimum allele frequency to at least 20%.
- See "Recommended materials" on page 14 for the recommended RNA isolation and quantification kits.

Copy number determination by qPCR

- If your qPCR data give a different relationship between C_t and copy number, this is likely a result of differences in the baseline or threshold selected. Determine the copy number of a sample according to the known copy number in control reactions.
- We recommend basing copy number on the N Protein C_t value.
- If the N Protein C_t value is not accurate, use the S Protein or ORF1ab C_t values to determine copy number.
- The copy number is only an estimate.

Table 4 Approximate copy number to C_t conversion—TaqPath™ COVID-19 RT-PCR kits

Tier	Viral copy number	TaqPath™ C_t		
		N Protein	S Protein	ORF1ab
Low	50–1,500	25–29	24–29	24–29
Medium	1,500–50,000	20–25	19–24	19–24
High	50,000–1,500,000	15–20	14–19	15–19

Create a Sample Set

If you are using Torrent Suite™ Software version 5.16 or later, you must create a Sample Set.

Create a Sample Set manually

Create a Sample Set manually by entering sample information into the Torrent Suite™ Software without the use of an external CSV file. This method of entering sample information is useful for creating small Samples Sets.

To create a Sample Set manually, enter individual samples into the software, then create a new Sample Set and add your samples to it. Alternatively, you can add your new samples to an existing Sample Set.

For Sample Sets that contain numerous samples, you may want to import samples using a CSV file. For more information, see "Create a Sample Set by importing samples from a CSV file" on page 41.

1. Sign in to Torrent Suite™ Software.
2. In the **Plan** tab, click **Samples**, then click **Add or Update Sample Set/Samples**.

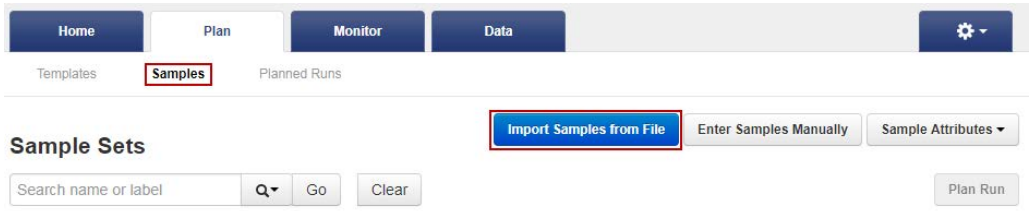
3. Click **Enter New Sample**.
   a. Complete the **Add Sample** dialog box. For information about defining the samples, see "Sample attributes" on page 71.
   b. Click **Done**.
      Your new samples and sample attributes appear in the **Enter Samples** list.
   c. Enter additional samples if needed.
4. Add the samples to a Sample Set. Do one of the following.
   - Click **Add to existing Sample Set**, then select an existing Sample Set to contain the samples.
   - Click **Create Sample Set**. For information about defining the Sample Set, see the Torrent Suite™ Software help system.
5. Click **Save Sample Set**.
   The set name appears in the **Sample Sets** list.

You can use the Sample Set to create a Planned Run. The information from the Sample Set and individual samples within the Sample Set are prepopulated in the Planned Run workflow bar steps and Planned Run template. For more information, see Torrent Suite™ Software help system.

Create a Sample Set by importing samples from a CSV file

If you have many samples, you can import new samples into Torrent Suite™ Software, or update existing samples, using a CSV file that contains sample information. If you do not yet have a samples file, you can create one from an available CSV template that is available in Torrent Suite™ Software. During this process, you can also create a new Sample Set for the new samples.

1. Sign in to Torrent Suite™ Software.
2. In the **Plan** tab, click **Samples**, then click **Import Samples from File**.



   If you do not yet have a samples file, create a new samples file from an available samples CSV file. For more information, see "Sample attributes" on page 71 and the Torrent Suite™ Software help system.
3. Upload the samples file, and optionally add a new Sample Set to receive the samples.
   a. In the **Import Samples** section, click **Select file**, then navigate to sample import file, then upload the sample import file.
   b. Select a Sample Set CSV file, then click **Open**.

c. Select a Sample Set to receive the samples.

(Optional) To add a new Sample Set to receive the samples, click **Add Sample Set**, then complete the Sample Set information.

For information about how to define a Sample Set, see Torrent Suite™ Software help system.

4. Click **Save & Finish**.

The system loads, parses, and validates the file, then, if no errors are found, saves the samples and Sample Sets.

Reverse transcribe RNA with the Ion Torrent™ NGS Reverse Transcription Kit

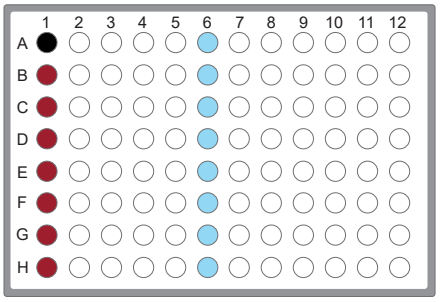
Prepare a master mix without sample RNA for multiple reactions plus 10% overage. Up to 8 sample libraries can be prepared in one Ion Chef™ Instrument run.

1. 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).

IMPORTANT! The reverse transcription mix is viscous. Pipet solutions slowly and ensure complete mixing by vortexing or pipetting up and down several times.

Component	Volume (10–µL/rxn)
Ion Torrent™ NGS 5X Reaction Buffer	2 µL
Ion Torrent™ NGS 10X RT Enzyme Mix	1 µL
Total RNA[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).



- Column 1 wells contain a 10 µL reverse transcription reaction, or control reaction—wells A1 to H1.
- *(Optional)* Positive control or no-template control (NTC)—well A1.
- 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—wells A6 to H6.

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 nontemplate control into column 1 wells that do not contain an RNA sample.
- Process at least 6 samples per run. If processing 5 or fewer samples, quantify the output combined library by qPCR to ensure that an optimal concentration is used in templating reactions.

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

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

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

STOPPING POINT Samples can be stored at 10°C for up to 16 hours in the thermal cycler. For longer term, store at –30°C to –10°C.

4. Briefly centrifuge the plate to collect the contents.

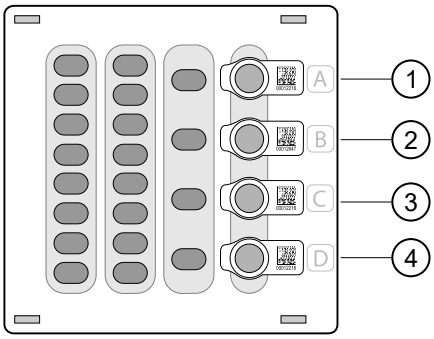
5. Add 5 µL of nuclease-free water to each sample, seal the plate with a MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge the plate to collect the contents. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

IMPORTANT! Before placing the plate on the Ion Chef™ Instrument, ensure that the plate has been sealed and centrifuged. This step is important to ensure that there is enough volume during the transfer to the pool 1 and pool 2 positions on the Ion Chef™ Instrument.

Prepare libraries on the Ion Chef™ Instrument

Abbreviated steps and panel-specific guidelines are provided here. See the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432) for detailed instructions for setting up the Ion Chef™ Instrument for library preparation using the Ion AmpliSeq™ Kit for Chef DL8.

1. Uncap all 4 tubes in positions A, B, C, and D in the Ion AmpliSeq™ Chef Reagents DL8 cartridge. Save the caps.



① Position A (150 µL Primer Pool 1 at 2X concentration)
② Position B (150 µL Primer Pool 2 at 2X concentration)
③ Position C (Empty tube)
④ Position D (Output tube)

2. After adding the primer pools, load the Ion AmpliSeq™ Chef Reagents DL8 cartridge, with uncapped tubes, in the Ion Chef™ Instrument, then load the following components.
   - Ion AmpliSeq™ Chef Solutions DL8 cartridge.
   - Ion AmpliSeq™ Tip Cartridge L8.
   - Enrichment Cartridge.
   - IonCode™ 96 Well PCR Plate in which you prepared cDNA. See “Reverse transcribe RNA with the Ion Torrent™ NGS Reverse Transcription Kit” on page 42. Remove the seal from the plate.
   - PCR Plate Frame.
   - PCR Frame Seal.

3. Set up the run on the Ion Chef™ Instrument following the instructions in *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide*. On the **AmpliSeq Workflow Options** screen, make the following selections.
   - **# of primer pools**—2
   - **Target amplification cycles**—The following cycle numbers are recommended.

Tier	Viral copy number	Number of amplification cycles
Low	50–1,500	27
Medium	1,500–50,000	21
High	50,000–1,500,000	16

   If the calculated viral copy number falls outside the ranges in the table, select the number of amplification cycles for the range closest to the calculated number.

Cycle number recommendations in the preceding table are based on qPCR quantification of viral copy number. Without qPCR quantification, use the following guidelines to determine optimal cycle number empirically.

- Low viral load suspected—27 cycles.
- High viral load suspected—21 cycles.
- Isolates or enriched viral particles—Approximately 16 cycles for 2 ng input.

- **Anneal & extension time**—4 minutes.

If you are working with samples with unknown viral load, and cannot quantify using qPCR, use 21 target amplification cycles as a starting point for library preparation on the Ion Chef™ Instrument.

4. After run setup, tap **Start Run** on the instrument screen.
   At completion of the run, the tube in Position D of the Ion AmpliSeq™ Chef Reagents DL8 cartridge contains 700 µL of combined barcoded libraries. We recommend that you quantify the concentration by qPCR using the Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802).

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

6 Create a Planned Run

IMPORTANT!

- This kit is compatible with Torrent Suite™ Software 5.12 or later. Before proceeding, we recommend that you update to the latest available versions of Torrent Suite™ and Ion Chef™ System software. Contact your service representative for help with upgrading the software.
- Ensure that you are using the latest version of the SARS_CoV_2_lineageID plugin (see "Update SARS_CoV_2_lineageID plugin online" on page 20).

Planned Runs

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

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

In Torrent Suite™ Software 5.12 or later, use the template as the primary Planned Run template for the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel.

Application category in Torrent Suite™ Software	Template name	Description
AmpliSeq DNA	`Ion_AmpliSeq_SARS-CoV-2-Insight.20210329.results_530.zip`	Planned Run template for the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel for the Ion 530™ Chip.
	`Ion_AmpliSeq_SARS-CoV-2-Insight.20210329.results_540.zip`	Planned Run template for the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel for the Ion 540™ Chip.

Create a custom Planned Run template

IMPORTANT! Before creating a Planned Run, you must import and enable the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel Run Plan templates. For more information, see “Before first use—Install plugins and Run Plan templates” on page 15. Contact your local service representative to obtain the most current BED files.

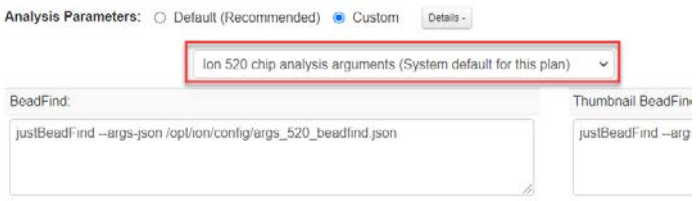
We recommend setting up a customized Planned Run template for reuse when the same conditions are expected to be used for multiple runs. For more information about creating Planned Runs manually or from the generic application template, see the Torrent Suite™ Software help system.

1. Sign in to the Torrent Suite™ Software.
2. In the **Plan** tab, in the **Templates** screen, click **AmpliSeq DNA** in the left navigation menu.
3. In the list of templates, find **Ion AmpliSeq SARS-CoV-2 Insight Research Panel-530** or **Ion AmpliSeq SARS-CoV-2 Insight Research Panel-540**, then click ⚙ ▸ **Copy**.

   IMPORTANT!
   - The **Ion AmpliSeq SARS-CoV-2 Insight Research Panel-530** template must be used with Ion 530™ Chip.
   - The **Ion AmpliSeq SARS-CoV-2 Insight Research Panel-540** template must be used with the Ion 540™ Chip.

   The **Copy Template** workflow opens to the **Save** step.
4. In **Template Name**, enter a name for the template.
5. For **Analysis Parameters**, select **Custom**.
6. In the dropdown list, select the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel (SARS plus) analysis arguments file for your chip.

   Import the analysis argument files before you create a Run Plan template. See “Import analysis argument file—Online” on page 18.



   The following information is automatically added to the **Alignment** and **Thumbnail Alignment** boxes.

```
tmap mapall ... -J 25 --end-repair 15 --context stage1 map4
```

   IMPORTANT! Do not manually change any parameters.

7. In the **Default Reference and BED Files** section, ensure that the correct Reference and Target Regions files are selected.

Entry or selection	Action
Reference Library	Ensure that `ion_ampliseq_sars-cov-2-insight(Ion Ampliseq Insight)` is selected.
Target Regions	Ensure that `Ion_AmpliSeq_SARS-CoV-2-Insight.20210329.designed.bed` is selected.
Hotspot Regions	Ensure that **None** is selected.

8. Click the **Ion Reporter** step, then ensure that **None** is selected.

9. In the **Research Application** step, verify that the **Research Application** is **DNA** and the **Target Technique** is **AmpliSeq DNA**, then click **Next**.

10. In the **Kits** step, verify that the **Ion Chef** is selected for the **Template Kit**, then complete the following items.

Item	Selection	
	Manual library preparation	Automated library preparation
Instrument	Ion GeneStudio™ S5 System	
Library Kit Type	Ion AmpliSeq™ Library Kit Plus	Ion AmpliSeq™ Kit for Chef DL8
Template Kit	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef or Ion 540™ Kit – Chef	
Sequencing Kit	Ion S5™ Sequencing Kit	
Chip Type	Ion 530™ Chip or Ion 540™ Chip	
Barcode Set	IonXpress or IonCode	IonCode
Flows	500	

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

12. In the **Plugins** step, ensure that following plugins are selected.
    - generateConsensus
    - SARS_CoV_2_coverageAnalysis
    - SARS_CoV_2_variantCaller
    - SARS_CoV_2_annotateSnpEff
    - SARS_CoV_2_lineageID

    For the SARS_CoV_2_variantCaller plugin, with Torrent Suite™ Software version 5.12, click **Configure**. In **Parameter settings**, ensure that the default setting, then click **Save Changes**.

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

14. In the **Save** step, click **Copy Template** to save the new Planned Run template.

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

Create a Planned Run for the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel

These instructions include the specific settings and selections required for a Planned Run that includes the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel. For more information, see Torrent Suite™ Software help system.

IMPORTANT! Use the correct template for your chip when creating a Run Plan.

- Use the Ion AmpliSeq SARS-CoV-2 Insight Research Panel-530 template with the Ion 530™ Chip.
- Use the Ion AmpliSeq SARS-CoV-2 Insight Research Panel-540 template with the Ion 540™ Chip.
- If you are using an Ion 550™ Chip, contact support for help.

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1. In Torrent Suite™ Software, in the **Plan** tab, in the **Templates** screen, select the correct template for your chip.

   The template was created in “Create a custom Planned Run template” on page 47.

2. In the **Ion Reporter** step, ensure that **None** is selected, then click **Next**.

3. In the **Research Application** step, ensure that the following parameters are selected, then click **Next**.
   - **Research Application**—**DNA**
   - **Target Technique**—**AmpliSeq DNA**

4. In the **Kits** step, ensure that the following settings are correct, then click **Next**.
   - **Chip Type**—Ion 530™ Chip or Ion 540™ Chip
   - **Barcode Set (optional)**—**IonXpress** or **IonCode**
   - **Template Kit**—**IonChef** button plus **Ion 510 & Ion 520 & Ion 530 Kit-Chef** or **Ion 540 Kit-Chef**
   - **Flows**—**500**

5. In the **Plugins** step, ensure that the following plugins are selected, then click **Next**.
   - **generateConsensus**
   - **SARS_CoV_2_coverageAnalysis**
   - **SARS_CoV_2_variantCaller**
   - **SARS_CoV_2_annotateSnpEff**
   - **SARS_CoV_2_lineageID**

6. In the **Projects** step, click **Next**.

7. In the **Plan** step, enter a name for the plan, then ensure that the following files are selected.

Note: The SARS_CoV_2_variantCaller loses optimized settings if a different BED file is selected.

File type	File
Reference Library	`ion_ampliseq_sars-cov-2-insight(Ion Ampliseq Insight)`
Target Regions	`Ion_AmpliSeq_SARS-CoV-2-Insight.20210329.designed.bed`

We recommend that you allow 500,000–1,000,000 reads per sample for the Ion AmpliSeq™ SARS-CoV-2 Research Panel. Use the following chip capacity table to set the maximum number of sample libraries loaded per chip.

Chip type	Reads per chip
Ion 530™ Chip	15–20 M
Ion 540™ Chip	60–80 M

8. Review the **Summary** pane, then click **Plan Run**.

You need to create a Planned Run for each chip that you load in an Ion Chef™ templating run.

Create a Planned Run for automated library preparation using Sample Sets

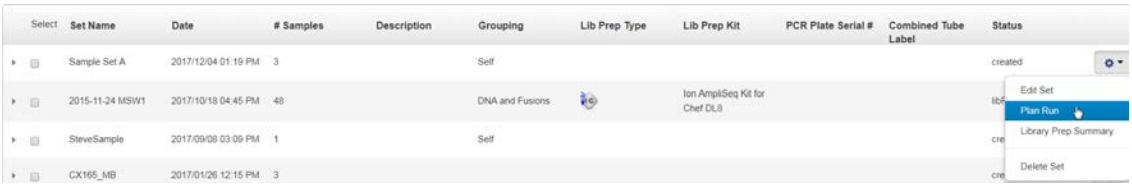
Automated Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel library preparation on the Ion Chef™ System requires use of Sample Set for creating a planned run. For more information on creating a Sample Set, see “Create a Sample Set” on page 40.

If combining more than one sample set, each sample set must correspond to Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel automated library preparations and use the same barcode kit to be included in a single Planned Run.

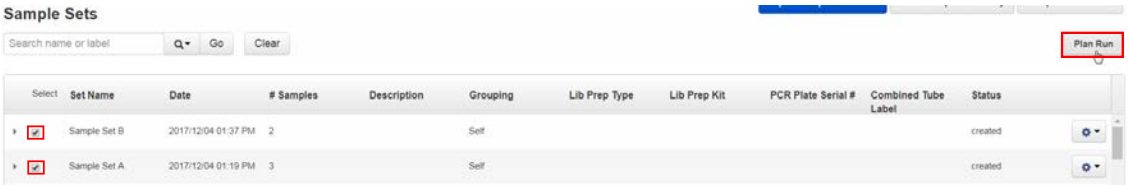
1. In the **Plan** tab, in the **Samples** screen, find the Sample Set or Sample Sets that you want to add to the Planned Run.

2. Select one or more Sample Sets to add to the Planned Run.

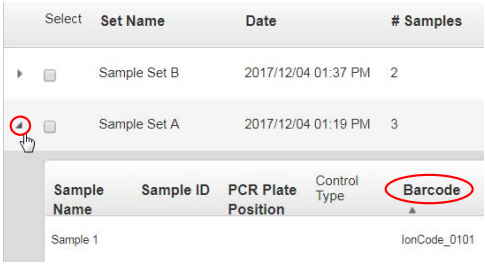
   - To plan a run using one Sample Set, click ⚙ **(Actions)** ▸ **Plan Run** in the row of the Sample Set.



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



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



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

IMPORTANT!

- An Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel—530 template must be used with the Ion 530™ Chip.
- An Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel—540 template must be used with the Ion 540™ Chip.

3. In the **Barcoding** step in the workflow bar, make the appropriate selections.

Item	Action
Analysis Parameters	Ensure that **Default (Recommended)** is selected.
Use same reference & BED files for all barcodes	Ensure that this checkbox is selected.
Same sample for DNA and Fusions?	Ensure that this checkbox is selected.
Number of barcodes	Enter the number of barcodes, then click ✔.
Sample Tube Label	Enter or scan the barcode of each Ion Chef™ Library Sample Tube to be used in the run.
Chip Barcode	No entry is required.
Oncology	Ensure that this is deselected.
Preimplantation Genetic Screening	Ensure that this is deselected.

4. Ensure that your Sample Set information has been correctly imported. If needed, edit entries in the **Samples Table**, then click **Next**.

Item[1]	Description
Barcode	Use the imported barcode selections or select the barcode for each sample from the dropdown list.
Sample Name (required)	Each sample must have a unique name. Type in the field to overwrite the default name. Use unique names even between runs.
Control Type (expanded)	Click the **Control Type** column heading to expand the **Control Type** column. Select **No Template Control** from the dropdown list to designate a sample as a no template control.
Sample ID	*(Optional)* Enter a sample ID.
Sample Description	*(Optional)* Enter a sample description.
Nucleotide type	Select **DNA** for each sample.
Annotations (expanded)	Not applicable—Leave blank.
Cancer Type	Not applicable—Leave blank.
Cellularity %	Not applicable—Leave blank.
Ion Reporter Workflow	Ensure that **None** is selected.
Relation	Not applicable—Leave blank.
Gender	Select **Male**, **Female**, or **Unknown**. Click  to copy the entry to all the rows.
IR Set ID	Not applicable—Leave blank.

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

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

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

7. In the **Save & Finish** step in the workflow bar, enter a name for the Planned Run in the provided field.

8. Click **Save & Finish**.

The run is listed in the **Planned Runs** screen under the name that you specified. The run is used by the Ion Chef™ System.

7 Guidelines for templating and sequencing

Proceed to template preparation and sequencing using the following kits.

Chip	Maximum libraries per chip	Kit	User guide
Ion 530™ Chip[1,2]	• 16[3] • 32[4]	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef (Cat. No. A34461)	*Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide* (Pub. No. MAN0016854)
Ion 540™ Chip[1,2]	• 64[3] • 128[4]	Ion 540™ Kit – Chef (Cat. No. A30011)	*Ion 540™ Kit – Chef User Guide* (Pub. No. MAN0010851)

[1] Template system: Ion Chef™ System

[2] Sequencer: Ion S5™ XL Sequencer, Ion GeneStudio™ S5 Plus Sequencer, or Ion GeneStudio™ S5 Prime Sequencer

[3] 1,000,000 reads per library

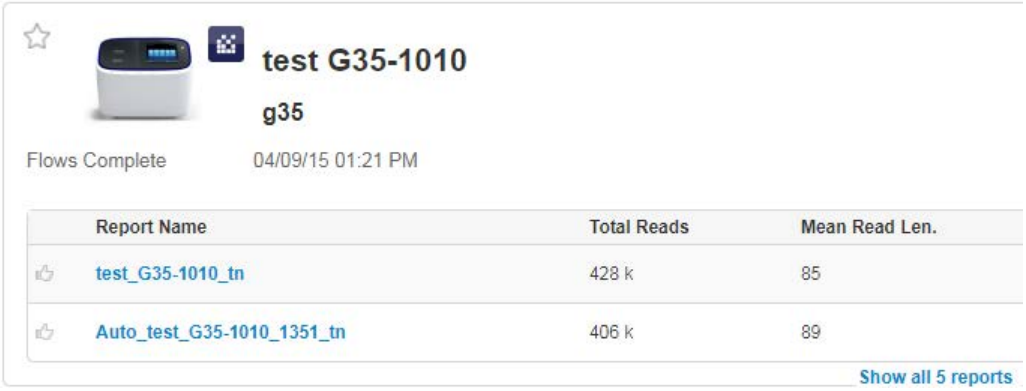
[4] 500,000 reads per library

8 Data analysis

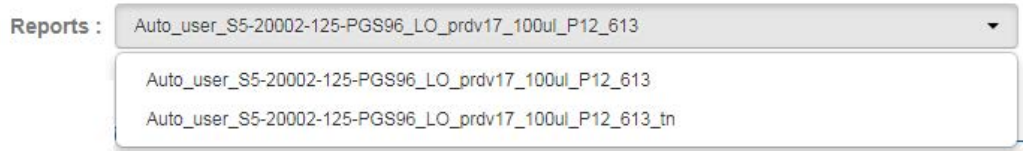
Open a run report

- Every sequencing run report includes at least two versions—a full chip report that contains complete details about the run, and a thumbnail version that includes a limited data set. If the run has been reanalyzed, additional reports are available.
- The thumbnail reports use a limited set of data points to give a summary of the fully sequenced and analyzed run. Thumbnail reports can be used to determine quickly the success of the run or whether a run should be terminated. Thumbnail reports are appended with `_tn` at the end of the report name and can be opened before the full chip reports are generated. The full chip reports contain all the data points and can be used for your analysis review.
- For details about run reports, see the Torrent Suite™ Software help system.

1. In the **Data** tab, click **Completed Runs & Reports**.
   Completed Runs & Reports are displayed in **List View** layout.
2. *(Optional)* Click **Table View** to review **Completed Runs & Reports** details in a table layout.
3. Open an individual run report.
   - In the **List View**, find the pane that contains details about your run of interest, then click the link in the **Report Name** column to view details about any of the available reports for that run.



   If more than two reports are listed, click **Show all reports** to view the list of reports associated with your run of interest.
   - In the **Table View**, find the run that you want to view the report for, then click the link in the **Report Name** column.
     To open another report that is associated with your run of interest, click the **Reports** list, then select the report that you want to open.



Check the sequencing run

Use the parameters in the following table to check the sequencing run.

Even if the values are not optimal, the run could still provide useful sequencing data.

Parameter	Optimum	Source
`Mean Read length`	≥175 bp	Torrent Suite™ Software—run report
`Mean Depth (MD)` and `Uniformity` (U)	• >1000 (MD) • >90% (U)	Torrent Suite™ Software—SARS_CoV_2_coverageAnalysis plugin section
`Genome Coverage`	≥99% of the genome covered at > 20X	Torrent Suite™ Software—SARS_CoV_2_coverageAnalysis plugin section

Open a plugin log

If a Plugin report indicates that an error occurred during a plugin run, you can view a log that contains details about the plugin run.

1. In the **Data** tab, click **Completed Runs & Reports**, then click the **Report Name** link for your completed sequencing run.
2. In the left navigation menu, click **Plugins**, or the name of the plugin that has the log you want to view.
3. Click **View Log** to the right of the plugin name.
   The log for the plugin run opens.

Delete plugin results from a run report

You can delete plugin results from the **Plugins** section of the run report.

1. In the **Data** tab, click **Completed Runs & Reports**, then click the report name link for your completed sequencing run.
2. In the left navigation menu, click **Plugins**, or the name of the plugin results that you want to delete.
3. Click **Delete** to the right of the plugin name.
   The plugin results are deleted from the run report.

Review SARS_CoV_2_coverageAnalysis plugin results

The SARS_CoV_2_coverageAnalysis plugin generates a Coverage Analysis Report. This report includes read statistics and several charts. The statistics and charts that are presented depend on the library type for the analysis.

The report summary lists the barcodes, the samples, the number of mapped reads, the percentage of on target reads, mean base coverage depth, and base coverage uniformity. Microsoft™ Excel™-compatible reports are also generated, including differential expression tables. Additional details regarding read coverage are also provided on a per-barcode basis, along with a list of gene annotations for each sequenced region.

You can download statistics files and the aligned reads BAM file from the file links at the bottom of the Coverage Analysis Report. After the sequencing run completes, review the plugin results in the report summary.

1. In the **Data** tab, click **Completed Runs & Reports**.
2. In the list of runs, find the run of interest, then click the link in the **Report Name** column.
3. In the left navigation menu, click **SARS_CoV_2_coverageAnalysis** to view the plugin summary.
   A summary table of the coverage analysis, by barcode, is included in the SARS_CoV_2_coverageAnalysis summary pane.

4. In the SARS_CoV_2_coverageAnalysis barcode summary, in the **Barcode Name** column, click a link to open a detailed **Coverage Analysis Report** for that barcoded sample.

   Alternatively, click the **coverageAnalysis.html** link to open the summary table for all barcodes in a new window.

5. In the **Coverage Analysis Report**, review the plugin results. Click the links at the bottom of the **Coverage Analysis Report** to download associated statistics and summary files for each barcoded sample in the run.

Review SARS_CoV_2_variantCaller plugin run results

After a SARS_CoV_2_variantCaller plugin run completes, you can access SARS_CoV_2_variantCaller run results from the run report screen in Torrent Suite™ Software. On the run report screen, in the SARS_CoV_2_variantCaller section you can do the following.

- Review the summary of the SARS_CoV_2_variantCaller plugin run for each barcode used.
- Review the library type, reference genome, targeted regions, hotspots file, and parameter settings that were used in the run.
- Download data files for all barcodes and each individual barcode or sample.

You can also access the detailed SARS_CoV_2_variantCaller plugin summary report for each barcode or sample from the SARS_CoV_2_variantCaller section on the run report screen. In the detailed SARS_CoV_2_variantCaller plugin summary report screen, you can do the following.

- View variant call information by allele for the specific barcode, such as allele location on the chromosome, allele annotations, coverage metrics, and quality metrics.
- Review the library type, reference genome, target regions, hotspots, and parameter settings that were used in the run.
- Download BED files and the parameters file that are used for the specific barcode.
- Download BAM and BAI files for the mapped and TVC-processed reads.
- Download data files for variant calls and coverage for the specific barcode.
- View variant calls in IGV.

The SARS_CoV_2_variantCaller plugin supports SNPs, MNPs, INDELs, and complex alleles as input candidates at genomic positions with the target regions file. If the variant is outside of the target regions, then the variant is not generated as a candidate and is not further evaluated, even if the variant is specified in the hotspots file.

For details, see “SARS_CoV_2_variantCaller plugin” on page 73 or the Torrent Suite™ Software help system.

1. In the **Data** tab, click **Completed Runs & Reports**.

2. In the **Completed Runs & Reports** list, find the run of interest, then click the report link in the **Report Name** column in the row of the run.

3. In the left navigation menu, click **SARS_CoV_2_variantCaller** to navigate to the SARS_CoV_2_variantCaller results section.
   - The plugin report includes a list of the barcodes that were used and file download options for all barcodes and each individual barcode.

   Table 5 Downloads options for all barcodes that were used in the plugin run.

Download option	Description
VCF.ZIP	A compressed folder that contains separate VCF files for each barcode.
XLS.ZIP	A compressed folder that contains separate XLS files for each barcode.
XLS	A file that contains a list of alleles for all barcodes in a tab-separated file that can be opened by Microsoft™ Excel™.
COV	A file that contains the coverage of the variant call results for all barcodes in tab-separated file format, which can be opened in Microsoft™ Excel™.

4. To open the detailed variantCaller plugin report for a specific barcode or sample, do one of the following in the variantCaller run summary table.
   - In the **Barcode name** column in a barcoded sequencing run report, click the barcode name link.
   - In the **Sample** column in a report from a sequencing run that does not contain barcodes, click the sample name link.

Review SARS_CoV_2_annotateSnpEff plugin results

The SARS_CoV_2_annotateSnpEff plugin generates an annotated list of variants. You can use this plugin to identify and annotate variants with public or private databases and perform multi-sample comparisons.

Additional details, including definitions about parameters, can be found at **https://pcingola.github.io/SnpEff/se_outputsummary/**.

1. In the **Data** tab, click **Completed Runs & Reports**, then in the **Report Name** column, click the report name link for the completed sequencing run.
2. In the left navigation menu, click **SARS_CoV_2_annotateSnpEff**.
3. Click a barcode to see the variant lists with SnpEff annotations.

   The SnpEff summary contains statistics about the variants.
4. To see a SnpEff report for a barcode, click **Review** in the **SnpEff Report** column or click the barcode, then click **snpEff_summary.html**.

   An HTML file of the results opens in a new browser window.
5. To download a ZIP file with summary reports for all variants, scroll to the bottom of the section, then click **SARS_CoV_2_annotateSnpEff.zip**.

   The ZIP file contains annotated variants in VCF output files for each barcode.

Review generateConsensus plugin results

The generateConsensus plugin generates FASTA files that contain the consensus sequence for each barcode. The SARS_CoV_2_lineageID plugin identifies low-frequency variants for highly variable RNA viruses (see “Review SARS_CoV_2_lineageID plugin results” on page 59).

To change the FASTA sequence file header, see “Run the generateConsensus plugin” on page 62.

1. In the **Data** tab, click **Completed Runs & Reports**, then in the **Report Name** column, click the report name link for the completed sequencing run.
2. In the left navigation menu, click **generateConsensus**.
   The screen scrolls to the **generateConsensus** section.
3. To download the FASTA file, click **Download Sample Consensus Sequences FASTA** .
4. To open the **generateConsensus** Report, click the **generateConsensus.html**.
5. To see the statistics for a library, click a barcode.

Review SARS_CoV_2_lineageID plugin results

The SARS_CoV_2_lineageID plugin assigns a lineage to a barcode using Pangolin software.

The version of Pangolin software is displayed towards the top of the SARS_CoV_2_lineageID section. The SARS_CoV_2_lineageID is regularly updated to align with updates to Pangolin software. To analyze results using another version of Pangolin software, contact support.

To see the definition of a parameter, place the pointer over the column title.

1. In the **Data** tab, click **Completed Runs & Reports**.
2. In the list of runs, find the run of interest, then click the link in the **Report Name** column.
3. In the left navigation menu, click **SARS_CoV_2_lineageID** to view the plugin summary.
   A summary table of the coverage analysis, by barcode, is included in the SARS_CoV_2_lineageID summary pane.
4. To view Lineage results for a barcode, click the Lineage for that barcode.
5. To see the consensus sequence, click the sequence in the **Consensus Sequence** column.
6. Click the link at the bottom of the **Download Barcode Summary Report** to download a CSV file with barcode summary results.

View troubleshooting and FAQs online

Visit our online Support Centers and FAQ database for tips and tricks for conducting your experiment, troubleshooting information, and FAQs. The online FAQ database is frequently updated to ensure accurate and thorough content.

- For the Next–Generation Sequencing Support Center: **http://thermofisher.com/ngssupport**
- For FAQs for this product: **http://thermofisher.com/A51305faqs**
- To browse the FAQ database and search using keywords: **thermofisher.com/faqs**

B

Manually run the SARS-CoV-2 plugins after the sequencing run is complete

After the sequencing run is complete, you may want to reconfigure the plugin settings and manually run the plugins.

For details about running the IRMAreport and AssemblerTrinity plugins, see the *Ion AmpliSeq™ SARS-CoV-2 Research Panel on an Ion GeneStudio™ S5 Series System Quick Reference* (Pub. No. MAN0019277).

IMPORTANT! You must manually run plugins in order.

Run the SARS_CoV_2_coverageAnalysis plugin

Follow these steps to run the SARS_CoV_2_coverageAnalysis plugin after the sequencing run is complete.

1. In the report summary for the completed sequencing run, click **Plugins** ▸ **Select Plugins to Run**, then click **SARS_CoV_2_coverageAnalysis**.
2. To view default settings, click **Show Advanced Options**.
3. Confirm or edit **Tier 1 Coverage Depth**, **Tier 2 Coverage Depth**, and the **Tier 3 Coverage Depth**.
4. Click **Submit**.

Run the SARS_CoV_2_variantCaller plugin

You can run the SARS_CoV_2_variantCaller plugin on a completed run report in Torrent Suite™ Software.

1. In the **Data** tab, click **Completed Runs & Reports** screen, then click the **Report Name** link for the completed sequencing run of interest.
2. Click **Plugins** ▸ **Select Plugins to Run**, then click the name of the plugin that you want to run.
3. In the **Select a Plugin to Run** dialog box, select the SARS_CoV_2_variantCaller plugin.
   The **Configure Plugin** window opens.
4. *(Optional)* Change any parameters.

   IMPORTANT! Before changing parameters, we recommend contacting support.
5. Click **Submit**.
   The SARS_CoV_2_variantCaller plugin reruns, then applies the changes that you made.

Run the generateConsensus plugin

Follow these steps to run the generateConsensus plugin after the sequencing run is complete. You can select some barcodes for a new analysis and change the configuration settings, if desired.

1. In the report summary for the completed sequencing run, click **Plugins** ▸ **Select Plugins to Run**, then click **generateConsensus**.
2. *(Optional)* In the **Configure Plugin** dialog box, make custom configuration changes.
   a. Select the **variantCaller Run** from the dropdown.
      If no selection is made, then the last variantCaller output is used.
   b. Select the file for **Barcode to FastaHeader Mapping**. The file is a CSV file composed of two columns—the barcode column and the FASTA header line column.
      - A FASTA file is generated to include only the barcodes listed in the CSV file and the header of each FASTA entry would be the one provided by the CSV file.
      - The FASTA header line column must comply with the required format of the public repository to which the FASTA is being deposited.
      - If no selection is made a FASTA file includes all barcodes used in the sequencing run.
   c. To change the read depth, click **Show advanced options**, then enter the minimum read depth.
      Before changing any advanced settings, contact support.
      The default setting is 20. Bases with read counts below the threshold are converted to *N* in the FASTA file.
3. Click **Submit**.

Run the SARS_CoV_2_lineageID plugin

Follow these steps to run the SARS_CoV_2_lineageID plugin after the sequencing run is complete. You can select some barcodes for a new analysis and change the configuration settings, if desired.

1. In the report summary for the completed sequencing run, click **Plugins** ▸ **Select Plugins to Run**, then click **SARS_CoV_2_lineageID**.
2. In the **Configure Plugin** dialog box, select the **generateConsensus Run**, then click **Submit**.

Run the SARS_CoV_2_annotateSnpEff plugin

Follow these steps to run the SARS_CoV_2_annotateSnpEff plugin after the sequencing run is complete.

The SARS_CoV_2_variantCaller plugin provides input for the SARS_CoV_2_annotateSnpEff plugin. The SARS_CoV_2_variantCaller plugin analysis must be complete before you run the SARS_CoV_2_annotateSnpEff plugin.

1. In the report summary for the completed sequencing run, in the left navigation menu, click **SARS_CoV_2_variantCaller**.
2. Copy the **SARS_CoV_2_variantCaller ID**, which is the number in parentheses next to the SARS_CoV_2_variantCaller version.
3. Click **Plugins** ▸ **Select Plugins to Run**, then click **SARS_CoV_2_annotateSnpEff**.
4. In the **SARS CoV-2 Annotate SnpEff Plugin** dialog box, select the **variantCaller Run**.
5. Click **Submit**.

C Supplemental procedures and information

Alternate reverse transcription protocol

This protocol should be used with dilute samples.

Using this protocol results in fewer reactions per Ion Torrent™ NGS Reverse Transcription Kit.

Reverse transcribe RNA with the Ion Torrent™ NGS Reverse Transcription Kit

1. Combine the following components per reaction to make a master mix for the total number of reactions, plus 10% overage.

Component	Volume (15–µL/rxn)
Ion Torrent™ NGS 5X Reaction Buffer	3 µL
Ion Torrent™ NGS 10X RT Enzyme Mix	1.5 µL
Total RNA[1]	≤10.5 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).

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

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

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

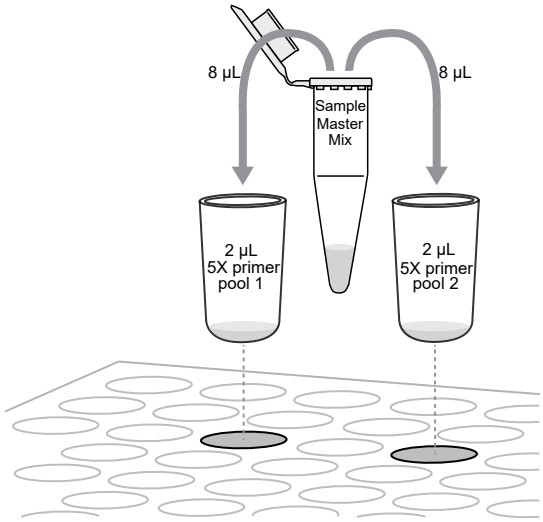
STOPPING POINT Samples can be stored at 10°C for up to 16 hours in the thermal cycler. For longer term, store at –30°C to –10°C.

Prepare cDNA target amplification reactions

1. Set up an amplification master mix for each sample using the entire volume of the reverse transcription reaction.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	4.5 µL
Reverse transcription reaction from “Reverse transcribe RNA with the Ion Torrent™ NGS Reverse Transcription Kit” on page 64	13.5 µL
Total volume	18 µL

2. Mix thoroughly by pipetting up and down 5 times, then transfer 8 µL of each sample-specific master mix into 2 wells of a 96-well PCR plate.



3. Add 2 µL of primer pool 1 into the first well, and 2 µL of primer pool 2 to the second well for a total of 10 µL in each well.

4. Seal the plate with a MicroAmp™ Clear Adhesive Film, place a MicroAmp™ Optical Film Compression Pad on the plate, then place the sealed plate in a thermal cycler.

   To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

Proceed to "Prepare cDNA target amplification reactions" on page 25.

Quantify the amplified library with a Qubit™ Fluorometer

IMPORTANT! Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel libraries must be amplified before quantification to enrich amplifiable material and obtain sufficient material for accurate quantification.

Amplify the library using 1X Library Amp Mix, then purify. Quantify the library using a Qubit™ 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 ~100 pM, which is appropriate for template preparation using an Ion template kit.

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

Amplify the library

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

   The 1X Library Amp Mix and 25X Library Amp Primers can be combined before addition.

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

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

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

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

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

Purify the amplified library

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

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

IMPORTANT!

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

First-round purification

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

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

Second-round purification

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

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

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

   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 briefly centrifuge to collect the contents. Alternatively, mix by setting a pipettor to 40 µL, then pipet the mixture up and down at least 5 times before sealing the plate.

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

10. Place the plate in the magnet for at least 2 minutes, then analyze an aliquot of the supernatant as described in "Qubit™ Fluorometer: Quantify the library and calculate the dilution factor" on page 68.

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

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

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

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

2. Based on the calculated library concentration, determine the dilution that results in a concentration of ~100 pM:

   Library concentration (pM) = 1660 x (Qubit™ concentration, ng/µL) + 480

3. Dilute library to the appropriate concentration.

Chip	Concentration
Ion 530™ Chip	30 pM
Ion 540™ Chip	50 pM

4. Combine libraries, then proceed to template preparation, or store libraries as described below.

(Optional) Combine amplicon libraries

Multiple libraries can be combined for sequencing on the same chip.

- Ion 530™ Chip: 16–32 libraries per chip
- Ion 540™ Chip: 32–128 libraries per chip

Store libraries

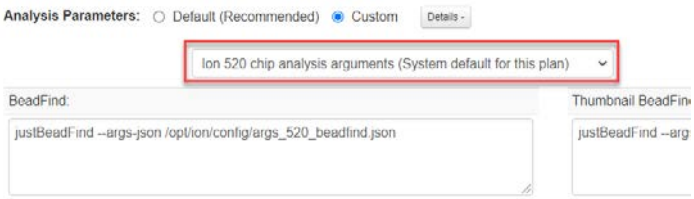
Store libraries at 4–8°C for up to 1 month. For longer lengths of time, store at –30°C to –10°C.

Create a custom Planned Run template—Ion 550™ Chip

IMPORTANT! Before creating a Planned Run, import and enable the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel Run Plan templates. For more information, see “Before first use—Install plugins and Run Plan templates” on page 15. Contact your local service representative to obtain the most current BED files.

We recommend that you set up a customized Planned Run template for reuse when the same conditions are expected to be used for multiple runs. For more information about creating Planned Runs manually or creating from the generic application template, see the Torrent Suite™ Software help system.

1. Sign in to the Torrent Suite™ Software.
2. In the **Plan** tab, in the **Templates** screen, click **AmpliSeq DNA** in the left navigation menu.
3. In the list of templates, find **Ion AmpliSeq SARS-CoV-2 Insight Research Panel-540**, then click ⚙ ▸ **Copy**.
   The **Copy Template** workflow opens to the **Save** step.
4. In **Template Name**, enter a name for the template.
5. For **Analysis Parameters**, select **Custom**.
6. In the dropdown list, select the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel (SARS plus) analysis arguments file for your chip.
   The analysis argument files must be imported before creating a Run Plan template (“Import analysis argument file—Online” on page 18).



7. In **Alignment** and **Thumbnail Alignment**, enter the following code.

```
tmap mapall ... -J 25 --end-repair 15 --context stage1 map4
```

8. In the **Default Reference and BED Files** section, ensure that the correct Reference and Target Regions files are selected.

Entry or selection	Action
Reference Library	Ensure that `ion_ampliseq_sars-cov-2-insight(Ion Ampliseq Insight)` is selected.
Target Regions	Ensure that `Ion_AmpliSeq_SARS-CoV-2-Insight.20210329.designed.bed` is selected.
Hotspot Regions	Ensure that **None** is selected.

9. Click the **Ion Reporter** step, then ensure that **None** is selected.

10. In the **Research Application** step, verify that the **Research Application** is **DNA** and the **Target Technique** is **AmpliSeq DNA**, then click **Next**.

11. In the **Kits** step, verify that **Ion Chef** is selected for the **Template Kit**, then complete the following fields as described.

Item	Selection	
	Manual library preparation	Automated library preparation
Instrument	Ion GeneStudio™ S5 System	
Library Kit Type	Ion AmpliSeq™ Library Kit Plus	Ion AmpliSeq™ Kit for Chef DL8
Chip Type	Ion 550™ Chip	
Template Kit	Ion 550™ Kit – Chef	
Sequencing Kit	Ion S5™ Sequencing Kit	
Barcode Set	IonXpress or IonCode	IonCode
Flows	500	

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

13. In the **Plugins** step, ensure that following plugins are selected.
    - generateConsensus
    - SARS_CoV_2_coverageAnalysis
    - SARS_CoV_2_variantCaller
    - SARS_CoV_2_annotateSnpEff
    - SARS_CoV_2_lineageID

    For the SARS_CoV_2_variantCaller plugin, with Torrent Suite™ Software version 5.12, click **Configure**. In **Parameter settings** ensure that the default setting is correct, then click **Save Changes**.

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

15. In the **Save** step, click **Copy Template** to save the new Planned Run template.

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

Sample attributes

When you create a Sample Set, you enter sample attributes into the Torrent Suite™ Software, regardless of whether you enter attributes manually or by importing samples using a CSV file. Most of the sample information is optional except for **Sample Name**.

Attribute	Description
Sample Name (Required)	The unique name of the sample. Use any combination of alphanumeric characters, plus spaces, periods (.), hyphens (-), and underscores (_).
Sample External ID	*(Optional)* If you manage samples in an external system, for example, a Laboratory Information Management System (LIMS), enter the identifier from that system.
Barcode Kit	The name of the barcode kit used to make a library from the sample. The same barcode kit must be used for all samples in a Sample Set.
Barcode	The name of the specific barcode in the selected barcode kit. Assign a unique barcode to each sample in a Sample Set.
Sample ID	*(Optional)* A unique identification code (SampleID) for each barcode in a sample. This helps to track samples or possibly identify misassignment between samples and barcodes in a sequencing run. If you manage samples in an external system (for example, a LIMS), you can use the identifier from that system. This attribute cannot be changed. This attribute is optional in Torrent Suite™ Software.

(continued)

Attribute	Description
Control Type	The control type used when preparing the sample. If blank, the sample is not a control type sample.
Basic Annotations	
Description	Typically, one or two sentences that describe the sample.
Nucleotide Type	**DNA**
Sample Source	The source from which the sample is extracted.
Panel Pool Type	**Dual Pool**
Gender	*(Optional)* The biological gender of the sample. • **Female** • **Male** • **Unknown**
Type	*(Optional)* The relationship type for this sample. Type is used in conjunction with **Relationship Group**, described below. For example, a relationship group can contain two samples, one with a type of **Tumor** and another with a type of **Normal**.
Relationship Group	*(Optional)* Use **Relationship Group** to designate a group of multiple related samples within the same Sample Set. For example, DNA and RNA samples from the same sample must have the same **Relationship Group** number.
Extra Annotations (used for specialized applications, such as preimplantation genetic screening (PGS) research or oncology research)	
Sample Collection Date	The date that the blood sample was drawn.
Sample Receipt Date	The date that the laboratory received the blood sample.
Cancer Type	Not applicable—Leave blank.
Population	Not applicable—Leave blank.
Mouse Strains	Not applicable—Leave blank.
Cellularity %	Not applicable—Leave blank.
Biopsy Days	Not applicable—Leave blank.
Cell Number	Not applicable—Leave blank.
Couple ID	Not applicable—Leave blank.
Embryo ID	Not applicable—Leave blank.

SARS_CoV_2_variantCaller plugin

The SARS_CoV_2_variantCaller plugin calls single-nucleotide polymorphisms (SNPs), multiple nucleotide polymorphisms (MNPs), insertions, deletions, and block substitutions in a sample across a reference or within a targeted subset of that reference.

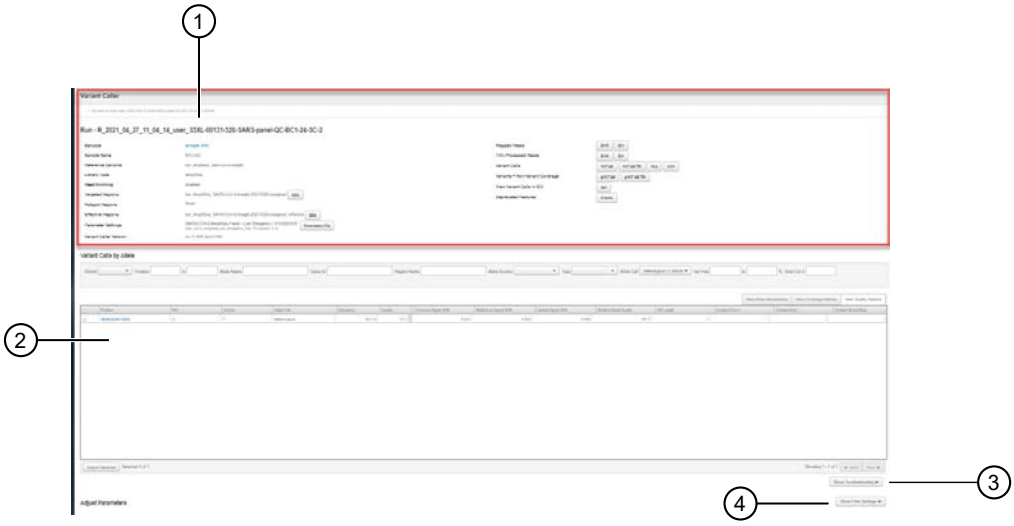
This plugin provides optimized preset parameters for many experiment types.

It can also be customized. After you find a parameter combination that works well on your data and that has the balance of specificity and sensitivity that you want, you can save that parameter set and reuse it in your research. Customization is supported when you run the plugin after a sequencing run and when the plugin is run through a Planned Run.

Detailed SARS_CoV_2_variantCaller plugin report

The detailed SARS_CoV_2_variantCaller plugin report contains run information, results, and the associated files for download that are specific for an individual barcode or sample.

To access the report, click the sample name link in the **Sample** column in a report from a sequencing run.



1. Review run information for a specific barcode or sample and download the associated files.
2. View variants called and their associated allele annotation information, coverage metrics, and quality metrics. For more information, see “Variant Calls by Allele table” on page 74.
3. Export the variant data files for troubleshooting. For more information, see the Torrent Suite™ Software help system.
4. Adjust variantCaller plugin filter settings that were used for the specific barcode or sample, then save the adjusted parameters to a new configuration. For more information, see the Torrent Suite™ Software help system.

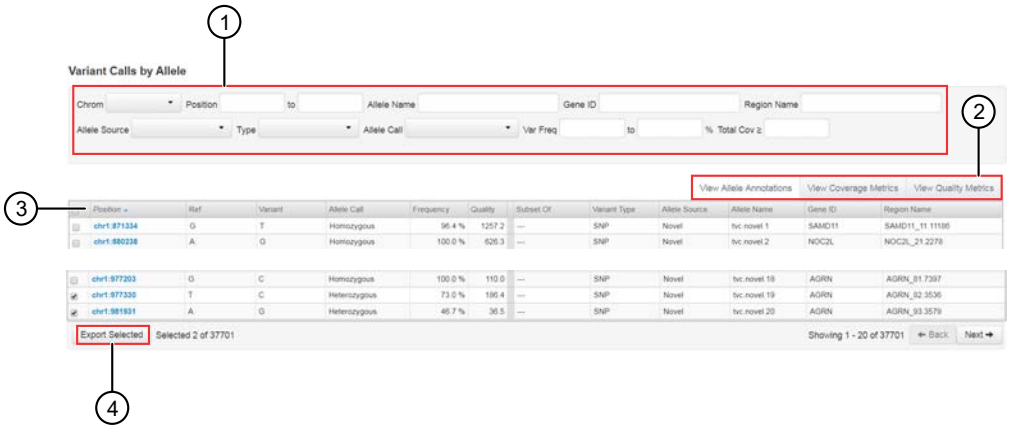
The following table lists and describes the download options for an individual barcode, or the sample used in the run. The available options in the table depend on the run type.

Download option	Description
Target Regions	**BED**—The BED file that specifies the genomic positions of interest.
Hotspot Regions	Not Applicable.
Effective Regions	**BED**—The BED file that specifies the regions that are processed in the SARS_CoV_2_variantCaller run for the sample or barcode (available if read trimming is enabled).
Parameter Settings	**JSON**—The JSON file that contains the parameter settings used in the SARS_CoV_2_variantCaller run for the sample or barcode.
Mapped Reads	• **BAM**—The BAM file that is input to the Torrent Variant Caller (TVC) module Pipeline for discovering and evaluating variants. Note that realignment may be applied. • **BAI**—The BAI file that contains the index information for the corresponding BAM file.
Torrent Variant Caller-Processed Reads	• **BAM**—The BAM file that is processed by Torrent Variant Caller (TVC) module . Note that read trimming and read filtering may be applied. In Tag Sequencing and Ion AmpliSeq™ HD runs, the BAM file may contain consensus reads that are obtained by compressing the reads in the mapped BAM that originate from the same DNA molecule. • **BAI**—The BAI file that contains the index information for the corresponding BAM file.
Variants Calls	• **VCF.GZ**—The compressed VCF file that contains the variant calls. • **VCF.GZ.TBI**—The index file for VCF.GZ. • **XLS**—The file that contains a list of variant alleles in the tab-separated file format, which can be opened in Microsoft™ Excel™. • **COV**—The file that contains coverage analysis for each variant call, which can be opened in Microsoft™ Excel™.
Variants + Non-Variant Coverage	• **gVCF.GZ**—The bgzip-compressed genome-VCF file that contains the variant calls. • **gVCF.GZ.TBI**—The tabix index file for gVCF.GZ.
View Variant Calls in IGV	**IGV**—The JNLP file that can be opened using (IGV) to visualize the variant calls.

Variant Calls by Allele table

The detailed SARS_CoV_2_variantCaller plugin report contains the **Variant Calls by Allele** table. The table lists the details about each variant that is called, including the allele locus, allele annotation, coverage metrics, and quality metrics for the specific barcode or sample. You can use the table to find the variant alleles of interest and information about those alleles. You can also export the information to be saved to your local storage.

To access the report, click the sample name link in the **Sample** column in a report from a sequencing run.



1. Find the variants of interest by applying filters to the table to narrow down the list of variants called.
2. Change the display of the table to view allele annotation, coverage metrics, or quality metrics for each variant. To switch between different displays, you can select one of the following tabs.
   - **View Allele Annotation**. For more information, see “Allele annotations ” on page 76.
   - **View Coverage Metrics**. For more information, see “View coverage metrics ” on page 77.
   - **View Quality Metrics**. For more information, see “View quality metrics” on page 78.
3. Click the column heading to sort variant alleles by the values in the column.
4. Export the information that is associated with the selected variant alleles to an XLS file. The exported XLS file contains all the information about the selected variants, including the information listed in the **View Allele Annotation**, **View Coverage Metrics**, and **View Quality Metrics** tabs. The tabs that are available depend on the run type. For information about how to export the information, see the Torrent Suite™ Software help system.

Column	Description
Position	The chromosome (or contig) name in the reference genome, and the position of the chromosome (or contig) in the one-based coordinate.
Ref	The reference base or bases.
Variant	The variant allele base or bases.
Allele Call	The zygosity (homozygous or heterozygous) or type (absent or No Call) of the allele that is called by the zygosity (homozygous or heterozygous) or type (Absent or No Call) of the allele all by the SARS_CoV_2_variantCaller plugin.
Frequency	The frequency, in %, of the variant allele.
LOD	Not applicable.

(continued)

Column	Description
Quality	The Phred-scored quality. For variants found by the Long INDEL Assembler, this value is always set to 50. Larger values mean higher confidence in the call. Quality is calculated by posterior probability that the variant allele frequency is greater than the cutoff (`min_allele_freq` in the parameter file), if a variant call is made, or posterior probability that the variant allele frequency is below this cutoff (if a reference call). The posterior probability that is computed as conditional on the reads observed includes sampling variability. Quality score is typically very large for reads strongly distinguishing variants with good depth, that is, under the model assumed, evidence is overwhelming for the variant or for the reference. Marginal values can mean that either the reads do not distinguish the variant well, there is insufficient depth to resolve, or the observed allele frequency is near the cutoff.
PPA (Optional)	An indication (0 or 1) of whether the variant allele is a possible polyploidy allele (PPA). Only absent alleles can be labeled as PPA; heterozygous and homozygous alleles are not treated as PPA. This column is available if the `report_ppa` parameter is set to 1. For more information on how to set the `report_ppa` parameter, see the Torrent Suite™ Software help system.

Allele annotations

You can view the following information in the **View Allele Annotations** tab of the **Variant Calls by Allele** table.

Column	Description
Allele Call	Decision whether the allele is detected (Heterozygous or Homozygous), not detected (Absent), or filtered (No Call). No Call and Absent are for only hotspot calls.
Subset Of	The name of the called allele that is a strict superset of the two SNPs. For example, if a called (homozygous or heterozygous) MNP is composed of two SNPs, then the MNP is considered to be a strict superset of the two SNPs.
Variant Type	The type of the variant called. • SNP—single nucleotide polymorphism. • IND—insertion. • DEL—deletion. • MNP—multiple nucleotide polymorphism or the substitution of a block sequence by the block of another length. • COMPLEX—Block substitution of sequence by a block of unequal length.
Allele Source	Allele source is called as: • Hotspot—for alleles included in the hotspots file. • Novel—for all other alleles.

(continued)

Column	Description
Allele Name	The allele name as defined in the hotspots file. For novel alleles, the name is defined as `tvc.novel.#`.
Gene ID	The Gene ID as defined in the target regions file.
Region Name	The region name as defined in the target regions file.

View coverage metrics

You can view the following information in the **View Coverage Metrics** tab of the **Variant Calls by Allele** table. The columns that are available on this tab vary according to the run type.

Column	Description
Total Read Cov	Total read coverage at this position, after downsampling. Variants calls are made on a sample of reads when coverage is higher than specified in the parameter settings file. This is referred to as "downsampling". For more information, see the help system for your version of Torrent Suite™ Software.
Read Cov +[1]	Total read coverage on the forward strand, after downsampling.
Read Cov -[2]	Total read coverage on the reverse strand, after downsampling.
Allele Read Cov	The number of reads that contain this allele, after downsampling.
Allele Read Freq[2]	The frequency of this allele across all reads.
Total Mol Cov[2]	The number of molecules covering this location.
Allele Mol Cov[2]	The number of detected molecules containing this allele.
Allele Mol Freq[2]	The frequency of molecules containing this allele.
Allele Cov +[3]	Allele coverage on the forward strand, after downsampling.
Allele Cov -[3]	Allele coverage on the reverse strand, after downsampling.
Strand bias[3]	The discrepancy between allele frequencies on the forward and reverse strands.

[1] This column is unavailable for sequencing runs that use Tag Sequencing as the target technique.
[2] This column is shown only for sequencing runs that use Tag Sequencing or Ion AmpliSeq™ HD as the target technique.
[3] This column is unavailable for sequencing runs that use Tag Sequencing or Ion AmpliSeq™ HD as the target technique.

View quality metrics

You can view the following information in the **View Quality Metrics** tab of the **Variant Calls by Allele** table.

This tab does not appear in Tag Sequencing runs.

Column	Description
Common Signal Shift	The distance between predicted and observed signal at the allele locus. [RBI]
Reference Signal Shift	The distance between predicted and observed signal in the reference allele. [REFB]
Variant Signal Shift	The distance between predicted and observed signal in the variant allele. [VARB]
Relative Read Quality	The Phred-scaled mean log-likelihood difference between the prediction under reference and variant hypothesis. [MLLD]
HP Length	Homopolymer length.
Context Error +	The probability of sequence-specific error on the forward strand (reported only for deletion variants).
Context Error -	The probability of sequence-specific error on the reverse strand (reported only for deletion variants).
Context Strand Bias	Basespace strand bias (reported only for deletion variants).

For candidates that are filtered out, the filtering reason is highlighted in the table.

lele coverage	allele coverage +	allele coverage -	strand bias
29	21	8	0.5897
23	15	8	0.5522
15	15	0	0.5000
15	15	0	0.5000
288	133	155	0.5000
95	88	7	0.5028
20	20	0	0.5000
5	0	5	0.5000
259	102	157	0.5000
187	80	107	0.5000
239	91	148	0.5000

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	Description
Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide (Pub. No. MAN0013432)	Describes the automated preparation and templating of Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel libraries using the Ion Chef™ System.
Ion AmpliSeq™ Library Kit Plus User Guide (Pub. No. MAN0017003)	Comprehensive instruction for the preparation of Ion AmpliSeq™ libraries and provides detailed instruction and troubleshooting for use of the Ion Library Equalizer™ Kit.
Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide (Pub. No. MAN0016854)	Describes the automated template preparation of Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel libraries using the Ion Chef™ System for sequencing on the Ion S5™ System.
Ion 540™ Kit – Chef User Guide (Pub. No. MAN0010851)	
Ion Library TaqMan™ Quantitation Kit User Guide (Pub. No. MAN0015802)	Provides detailed instruction and troubleshooting for use of the Ion Library TaqMan™ Quantitation Kit.
Demonstrated Protocol: Sample Quantification for Ion AmpliSeq™ Library Preparation Using the TaqMan™ RNase P Detection Reagents Kit (Pub. No. MAN0007732)	Provides detailed instruction for sample quantification using the TaqMan™ RNase P Detection Reagents Kit.
Torrent Suite™ Software 5.16 User Guide (Pub. No. MAN0019153)	Comprehensive instruction for use of Torrent Suite™ Software.

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

Customer and technical support

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

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- 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**.