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

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Note: For safety and biohazard guidelines, see the "Safety" appendix in the *Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay User Guide* (Pub. No. MAN0024915). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

This quick reference provides guidelines and instructions for manually preparing Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel libraries.

This quick reference is designed to be used by experienced users. For detailed instructions, see the *Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay User Guide* (Pub. No. MAN0024915).

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 Amplification 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.

Copy number determination by qPCR

Note:

- 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.

Approximate copy number to C_t conversion—TaqMan™ 2019-nCoV Assay Kit v1

Tier	Viral copy number	TaqMan™ C_t		
		N Protein	S Protein	ORF1ab
Low	50–1500	26–31	28–33	29–34
Medium	1500–50000	21–26	23–28	24–29
High	50000–1500000	16–21	18–23	19–24

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	50–1500	25–29	24–29	24–29
Medium	1500–50000	20–25	19–24	19–24
High	50000–1500000	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 the *Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay User Guide*.

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
Ion Torrent™ NGS 5X Reaction Buffer	2 µL
Ion Torrent™ NGS 10X RT Enzyme Mix	1 µL
Total RNA	≤7 µL
Nuclease-free Water	to 10 µL
Total volume per well	**10 µL**

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

3. Place a MicroAmp™ 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.

   We recommend using 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™ Compression Pad on the plate, then place the sealed plate in a thermal cycler.

Amplify the cDNA targets

Run the following program to amplify the target regions.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	98°C	2 min
Cycle; set number according to page 3	Denature	98°C	15 sec
	Anneal and extend	60°C	4 min
Hold	—	10°C	Hold

Recommended cycle number

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: ~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! Keep each plate on ice or in a prechilled 4°C cold block while preparing the reactions.

1. Combine primer pool 1 and 2 target amplification reactions.
2. Thaw the FuPa Reagent (brown cap) on ice, gently vortex to mix, then centrifuge briefly to collect.
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 clear adhesive film, vortex thoroughly, then centrifuge briefly to collect droplets.
5. Place a compression pad on the plate, load in the thermal cycler, then run the following program:

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

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 the *Ion AmpliSeq™ Library Kit 2.0 User Guide*.

Ion Xpress™ Barcode Adapters only: Combine and dilute adapters

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

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

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

[1] X = barcode chosen

Note: Store diluted adapters at –20°C.

Perform the ligation reaction

1. If there is visible precipitate in the Switch Solution or the tube cap after thawing, vortex or pipet up and down at room temperature to resuspend before pipetting.
2. If you have not already done so, briefly centrifuge the plate to collect the contents, then carefully remove the plate seal.

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

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

Order of addition	Component	Volume
1	Switch Solution (yellow cap)	4 µL
2	Adapters (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**	**~30 µL**

4. Seal the plate with a new MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.

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

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

Purify the unamplified library

1. Briefly centrifuge the plate to collect the contents in the bottom of the wells.

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

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.

6. Repeat step 5 for a second wash.

7. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes.

Quantify or normalize the libraries using the qPCR or equalizer kit. For details, see the *Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay User Guide* (Pub. No. MAN0024915).

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

Guidelines for templating and sequencing

Proceed to template preparation and sequencing using the following kits.

Chip	Maximum libraries/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
[4] 500,000 reads

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.

IMPORTANT! You must use the correct template for creating a run plan.

- Use the correct template for your chip.
- 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.
- If you are using an Ion 550™ Chip, contact support for assistance.

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

File type	File
Reference Library	ion_ampliseq_sars-cov2-insight (AmpliSeq SARS-CoV-2 Reference)
Target Regions	Ion_Ampliseq_SARS-CoV-2.2020323.Designed.bed

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

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**.

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

Limited product warranty

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

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Revision	Date	Description
B.0	11 June 2021	• Updated product name. • Corrected Ct conversion table for TaqPath™ kits. • Corrected cycling conditions for manual library preparation.
A.0	28 May 2021	New document for the Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel.