# Ion AmpliSeq™ SARS-CoV-2 Research Panel

Instructions for use on the Genexus™ Integrated Sequencer

Pub. No. MAN0019278 Rev. B.0

**WARNING!** Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

This quick reference provides guidelines and instructions for using the Ion AmpliSeq™ SARS-CoV-2 Research Panel to prepare Ion AmpliSeq™ libraries from SARS-CoV-2 samples and sequence the libraries on the Genexus™ Integrated Sequencer, then analyze the sequencing results using Genexus™ Software.



## Product description

The Ion AmpliSeq™ SARS-CoV-2 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 expression 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 potential serotypes. The panel is a community Ion AmpliSeq™ panel available for order through **AmpliSeq.com**.

When used in conjunction with the Genexus™ Integrated Sequencer, the Ion AmpliSeq™ SARS-CoV-2 Research Panel offers high sensitivity, high throughput (up to 16 samples per sample-to-result sequencing run), fast turnaround time, and minimal hands-on time in SARS-CoV-2 research studies.

## Ordering instructions

To order the Ion AmpliSeq™ SARS-CoV-2 Research Panel, follow these steps.

1. Go to **AmpliSeq.com** and sign in, or register for a new account.
2. In the navigation bar, go to the **Fixed Panels** dropdown menu, then select **Community Panels**.
3. In the **Research Area** navigation pane on the left side of the screen, select the **Infectious Disease** checkbox to filter the list. Find the Ion AmpliSeq™ SARS-CoV-2 Research Panel in the filtered list, then click **Preview Order**.

   **Note:** Alternatively, enter **SARS-CoV-2** in the search field at the top of the screen to find the panel page.
4. In the **Order options** window, select **Genexus** in the **Choose instrument** section, then click **Next**.
5. In the **Order summary** window, review the order, then select **Proceed to cart**. As an option, select the **List recommended consumables** checkbox, then click **Preview** to see a list of additional products that you may need. Select the items, then click **Add all to cart**.
6. Click **Proceed to checkout** to complete the order at **thermofisher.com**.

   Unless otherwise indicated, all other materials listed in this quick reference are available at **thermofisher.com**.

## Isolate and quantify viral RNA

### Guidelines for RNA isolation and sample normalization

- A sample containing as little as 20 copies of viral RNA (10 copies per target amplification reaction) can be used to prepare an Ion AmpliSeq™ SARS-CoV-2 Research Panel library. For optimal results, we recommend a viral copy number in the 200 to 200,000 range, or an amount of *total* RNA between 1–10 ng. For more information, see “Guidelines for sample quality, viral copy number, and variant calling” on page 5.
- The amount of viral RNA among samples should be approximately equivalent so that the target amplification conditions you select are optimal for all samples.
- See “Recommended materials for isolation and quantification” on page 2 for recommended Thermo Fisher Scientific kits and master mix.

## Recommended materials for isolation and quantification

We recommend the following Thermo Fisher Scientific kits and master mix for the isolation and quantification of SARS-CoV-2 RNA.

| Item | Cat. No. |
|---|---|
| **Isolation** | |
| MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit | A42352 or A48310 |
| **Quantification** | |
| TaqMan™ 2019-nCoV Assay Kit v1 | A47532 |
| TaqMan™ 2019-nCoV Control Kit v1 | A47533 |
| TaqPath™ 1-Step RT-qPCR Master Mix, CG | A15299 or A15300 |

Additional positive controls are available at the BEI Resources Repository at **https://www.beiresources.org**, or through other commercial providers.

## Isolate viral RNA

The MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit can be used in either a manual or a high-throughput automated mode using the MagMAX™ Express Magnetic Particle Processor or KingFisher™ Purification System. Follow these basic steps to isolate SARS-CoV-2 RNA using the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (manual extraction). For detailed information on how to use the kit, and required materials not supplied, see the following user guides, which are available for download at **thermofisher.com**.

- *MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (manual extraction) User Guide* (Pub. No. MAN0018072) or the
- *MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (automated extraction) User Guide* (Pub. No. MAN0018073)

1. Digest 200–400 µL of each sample with Proteinase K in a deep-well 96-well plate, then bind RNA to Nucleic Acid Binding Beads.
2. Wash the Nucleic Acid Binding Beads.
3. Elute the RNA from the Nucleic Acid Binding Beads.

Use 1–10 ng *total* RNA in library target amplification reactions. We recommend quantifying viral copy number by real-time PCR, described in "Quantify by real-time qPCR".

## Quantify by real-time qPCR

To determine the optimal system-installed SARS-CoV-2 assay parameter set to use for run planning in Genexus™ Software, quantify viral RNA copy number in samples following these steps and using the kits and mastermix listed in "Recommended materials for isolation and quantification". For more information about reaction set up, see the *TaqMan™ 2019-nCoV Assay Kit v1 Product Information Sheet* (Pub. No. MAN0019096).

After you quantify RNA viral copy number, or if you do not quantify copy number, follow the guidelines for selecting an assay in step 3 of "Plan a sample run with the Ion AmpliSeq™ SARS-CoV-2 Research Panel in Genexus™ Software" on page 3.

1. For each 2019-nCoV qPCR assay (N Protein, S Protein, and ORF1ab), combine the following components per reaction to make a reaction mix for the total number of reactions, plus 10% overage.

| Component | Volume per reaction |
|---|---|
| TaqPath™ 1-Step RT-qPCR Master Mix, CG (4X) | 6.25 µL |
| 2019-nCoV assay (20X; N Protein, S Protein, or ORF1ab) | 1.25 µL |
| RNAse P assay (20X) | 1.25 µL |
| RT-PCR Grade Water | 11.25 µL |
| **Total reaction mix volume** | **20.0 µL** |

2. For each reaction, combine the following components in a MicroAmp™ Optical 96-Well Reaction Plate 0.2-mL well.

| Component | Volume per well |
|---|---|
| Reaction mix (from step 1) | 20.0 µL |
| • Nucleic acid research sample *or*<br>• 1 µL 2019-nCoV Control v1 + 4 µL RT-PCR Grade Water *or*<br>• NTC | 5.0 µL |
| **Total reaction volume** | **25.0 µL** |

3. Set up and run the reactions on a real-time PCR instrument using the following settings:
   - Analysis method: Comparative $C_t$

     **Note:** You must use Comparative $C_t$ to analyze 2019-nCoV assay data using QuantStudio™ Design and Analysis Software v2 and ExpressionSuite™ Software.
   - Cycling mode: Standard
   - Thermal cycling protocol:

| Stage | Step | Temperature | Time |
|---|---|---|---|
| Hold | UNG incubation[1] | 25°C | 2 minutes |
| Hold | Reverse transcription | 50°C | 15 minutes |
| Hold | Activation[2] | 95°C | 2 minutes |
| Cycling (40 cycles) | Denaturation | 95°C | 3 seconds |
| | Anneal/ Extension | 60°C | 30 seconds |

[1] Heat-labile UNG in TaqPath™ 1-Step RT-qPCR Master Mix, CG is completely inactivated during the first ramp to 95°C.

[2] Required for RT inactivation, first denaturation, and activation of the DNA polymerase.

Use the $C_t$ result for each 2019-nCoV qPCR assay to estimate copy number in your sample. See "Copy number determination by qPCR" on page 3 for example data.

### 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.

**Copy number determination of SARS-CoV-2 with TaqMan™ 2019-nCoV Assay Kit v1 and TaqMan™ 2019-nCoV Control Kit v1**

| $C_t$ of N Protein | $C_t$ of S Protein | $C_t$ of ORF1ab | Copies in qPCR reaction |
|---|---|---|---|
| 34 | 36 | 37 | 5 |
| 33 | 35 | 36 | 10 |
| 32 | 34 | 35 | 20 |
| 31 | 33 | 34 | 39 |
| 30 | 32 | 33 | 78 |
| 29 | 31 | 32 | 156 |
| 28 | 30 | 31 | 312 |
| 27 | 29 | 30 | 625 |
| 26 | 28 | 29 | 1,250 |
| 25 | 27 | 28 | 2,500 |
| 24 | 26 | 27 | 5,000 |
| 23 | 25 | 26 | 10,000 |
| 22 | 24 | 25 | 20,000 |
| 21 | 23 | 24 | 40,000 |
| 20 | 22 | 23 | 80,000 |
| 19 | 21 | 22 | 160,000 |
| 18 | 20 | 21 | 320,000 |
| 17 | 19 | 20 | 640,000 |

## Create samples in Genexus™ Software

Before planning a sample run, you must create samples in the software. For detailed information on how to enter sample information into Genexus™ Software, see the *Genexus™ Integrated Sequencer User Guide* (Pub. No. MAN0017910).

## Plan a sample run with the Ion AmpliSeq™ SARS-CoV-2 Research Panel in Genexus™ Software

These instructions include specific settings and selections required for a run planned with the Ion AmpliSeq™ SARS-CoV-2 Research Panel. For detailed instructions for planning sample runs, see the *Genexus™ Integrated Sequencer User Guide* (Pub. No. MAN0017910).

Two system-installed SARS-CoV-2 assay parameter sets, the Ion AmpliSeq SARS-CoV-2 Research Assay and the Ion AmpliSeq SARS-CoV-2-LowTiter Research Assay, must be obtained in an update of the Genexus™ Software (6.2.0 or later) before you can plan a run with the panel.

1. In Genexus™ Software, click **Runs ▸ Plan Sample Run**.
2. In the **Setup** step, enter a unique name in the **Plan** section, then click **Next**.
3. In the **Assays** step, select the system-installed assay based on the viral copy number in samples (determined in "Quantify by real-time qPCR" on page 2), then click **Next**.

| Virus copy number per target amplification reaction | System-installed assay |
|---|---|
| ≤200 copies | Ion AmpliSeq SARS-CoV-2-LowTiter Research Assay |
| >200 copies, or virus titer not quantified | Ion AmpliSeq SARS-CoV-2 Research Assay |

4. In the **Samples** step, select the samples from the list that you want to run with the Ion AmpliSeq SARS-CoV-2 Research Assay, then click **Assign**.

   **IMPORTANT!** We recommend that viral copy titer between samples in a run differs no more than 500-fold (<9 $C_t$) to ensure that read counts for lower titer samples are adequate.

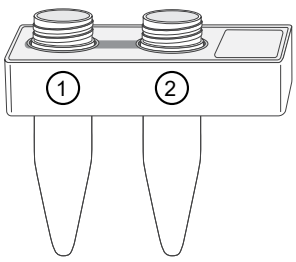
5. In the **Sample Plate** step, review sample positions in the sample plate. Modify the concentration of samples, if needed, then click **Next**.

   **IMPORTANT!** If a sample contains ≥500,000 viral copies per 25 µL based on real-time PCR quantification, we recommend that you adjust sample concentration to give a dilution factor of 10. If the nucleic acid concentration that you entered for the sample already results in a dilution factor ≥10, no further action is needed.

6. In the **Review** step, review the run plan summary, then click **Save & Print** to print the run setup guide, if desired. Click **Save** to save the run without printing.

## Fill Genexus™ Primer Pool Tubes

Genexus™ Primer Pool Tubes (Cat. No. A40262) must be manually filled with the Ion AmpliSeq™ SARS-CoV-2 Research Panel primer pools at the appropriate volume and in the correct primer pool tube position. Use one carrier per assay primer pool. The two positions in the primer pool tube carrier are designated as shown in the following figure:




① Position 1 tube: Contains Ion AmpliSeq™ SARS-CoV-2 Research Panel Primer Pool 1 or Pool 2.

② Position 2 tube: Leave empty.

1. Add 75 µL of each primer pool to the position 1 tube in as many primer pool tube carriers as specified by the run setup guide.

   The number of carriers used in a run depends on the number of samples in the run. In a run with 16 samples loaded, 4 carriers are needed for each primer pool in the panel.

| Number of primer pairs per pool | Concentration | Volume in position 1 | Volume in position 2 |
|---|---|---|---|
| 12-1,228 | 5X (250 nM) | 75 µL | — |

**IMPORTANT!**

- Leave the tube in position 2 empty and uncapped, but do not remove the tube from the carrier before loading in the sequencer. Do not add the second Ion AmpliSeq™ primer pool to the position 2 tube.
- Ensure that no bubbles are introduced at the bottom of the tube when adding the primer pool.

2. Load the uncapped Genexus™ Primer Pool Tubes in the sequencer according to the run setup guide.

   **Note:** If you do not install the primer pool tube carriers in the sequencer immediately, cap the tubes that contain primer pools, then store the tube carriers on ice. Remember to uncap all tubes before installing.

## Load the sample plate

1. Load 25 µL of each sample in the sample plate position specified in the run setup guide.

   **IMPORTANT!** If a sample contains ≥500,000 viral copies per 25 µL based on real-time PCR quantification, and the sequencer is not diluting the sample by a factor ≥10 automatically, we recommend that you manually dilute the sample 10-fold with nuclease-free water to reduce the titer to <200,000 copies per 25 µL.

   If the nucleic acid concentration that you entered for the sample in run planning results in a dilution factor ≥10, no further action is needed.

2. Seal the plate with a sheet of Adhesive PCR Plate Foils (Thermo Fisher Scientific Cat. No. AB0626).

3. Keep the plate on ice until you are ready to load it in the sequencer.

## Start a sequencing run

After you have planned a sample run in Genexus™ Software, loaded the panel in Genexus™ Primer Pool Tubes, and loaded the sample plate, set up the Genexus™ Integrated Sequencer for a run as described in the *Genexus™ Integrated Sequencer User Guide* (Pub. No. MAN0017910).

## Analyze SARS-CoV-2 sequencing results in Genexus™ Software

You can analyze the sequencing results in Genexus™ Software with SARS-CoV-2 plugins. Plugins included as part of the analysis are described in the following table.

**SARS-CoV-2 Genexus™ Software plugins**

| Plugin | Description |
|---|---|
| **COVID19AnnotateSnpEff** | Generates an annotated list of variants. You can use this plugin to identify and annotate variants with public or private databases, and enable multi-sample comparisons. |
| **IRMAreport** | Generates FASTA files of the consensus sequence for each barcoded sample. The Iterative Refinement Meta-Assembler (IRMA) identifies low-frequency variants for highly variable RNA viruses. |
| **AssemblerTrinity** | Generates a genome-guided or *de novo* viral sequence. The AssemblerTrinity plugin uses inchworm, chrysalis, and butterfly software modules sequentially, then builds de Bruijn graphs to resolve alternatively spliced isoforms and transcripts derived from paralogous genes to generate contig sequences. |
| **AssemblyStats** | Provides QC metrics (% sequence identity of sample to reference sequence) corresponding to each of the IRMAreport and AssemblerTrinity assembled sequences. |
| **ControlStat** | Provides QC metrics for the 5 human expression control targets included in the Ion AmpliSeq™ SARS-CoV-2 Research Panel, and metrics for the relative expression of SARS-CoV-2 and human control targets in a sample. |

**Other plugins included in Genexus™ Software for data analysis**

| Plugin | Description |
|---|---|
| **coverageAnalysis** | Used to view statistics and graphs that describe the level of sequence coverage produced for targeted genomic regions. |
| **CustomerSupportArchive** | Generates a downloadable archive that a technical support representative can use to troubleshoot and diagnose problems with sequencing runs or with Genexus™ Software. |

Review results from a completed SARS-CoV-2 GX run

1. In Genexus™ Software, click **Results ▸ Sample Results**.
2. In the **Sample Name** column, click the sample name of interest.
3. Click **Plugins**.
4. View **COVID19AnnotateSnpEff** plugin results.
   a. Scroll to the **COVID19AnnotateSnpEff** section to view the **COVID19AnnotateSnpEff Results** table.
   b. Click the **COVID19AnnotateSnpEff.zip** link to download a ZIP file that contains annotated variants in VCF output files for each barcode.
5. View **IRMAreport** plugin results.
   a. Scroll to the **IRMAreport** section to view the **IRMA Assembled Results** table.
   b. Click the **FASTA** link to view the consensus sequence for each sample.
6. View **AssemblerTrinity** plugin results.
   a. Scroll to the **AssemblerTrinity** section to view the **Trinity De novo Assembly Report Results** table.
   b. Click the **FASTA** links to view the individual contigs and the longest contig for each sample.
7. View the **AssemblyStats** plugin results.
   a. Scroll to the **AssemblyStats** section to view the **AssemblyStats** results table. The plugin displays the percent sequence identity of the **AssemblerTrinity** and **IRMAreport** assembled sequences to a SARS-CoV-2 reference sequence (Accession No. MN908947).
   b. Click the **AssemblerTrinity Alignment Result** and **IRMA Alignment Result** links to view the alignment of the query with reference sequence using each plugin.
8. View the **ControlStat** plugin results.
   a. Scroll to the **ControlStat** section to view the **ControlStat** results table.

   **ControlStat plugin metrics**

| Metric | Description |
|---|---|
| **Average Reads per Control Target** | The average number of reads per human expression control target (Total number of control reads / 5). |
| **Percentage of Library Reads** | The percentage of viral target reads over total reads. The percentage of human expression control reads = 100 – **Percentage of Library Reads**. |
| **Library/Control Reads per Target** | (Average reads per SARS-CoV-2 target) / (Average reads per human expression control target) —a measure of the relative amount of viral RNA in the sample compared to human RNA. |

   b. Click the links in the **Barcode Name** column to view the number of reads per expression control target associated with each individual barcode, and with both barcodes.
9. *(Optional)* Click ··· **(More Options) ▸ Download Files**, then select **DNA SmallVariants filtered vcf File** to download and view the VCF file that contains the variants that are analyzed with the COVID19AnnotateSnpEff plugin.

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

**Sample quality and viral copy number**

| Viral copy number | Recommendations and guidelines |
|---|---|
| 200 to 200,000 copies | Recommended range for optimal results |
| 120 to 199 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 *Genexus™ Software 6.2 User Guide* (Pub. No. MAN0018955). |
| 20 to 119 copies | Only for high-quality samples without degradation that contain a very small quantity of human RNA. Low-frequency, random, false-positives are likely due to errors occurring in reverse transcription. We recommend ignoring heterozygous variant calls from samples in this range. |

- 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 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%.

## Limited product warranty

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**Product:**
Genexus™ Software

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



**Revision history:** Pub. No. MAN0019278

| Revision | Date | Description |
|---|---|---|
| B.0 | 8 October 2020 | • Updated ordering instructions for AmpliSeq.com.<br>• Added new guidance for sample quality, viral copy number, and variant calling. |
| A.0 | 10 August 2020 | New quick reference for using the Ion AmpliSeq™ SARS-CoV-2 Research Panel on the Genexus™ Integrated Sequencer |