OSPHL 753 Virology / Immunology Ion AmpliSeq™ SARSCoV2 Insight Research Panel Library Prep

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Approval and Periodic Review Signatures

Type	Description	Date	Version	Performed By	Notes
Approval	Lab Director	8/11/2021	2.0	John Fontana John Fontana	
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2.0	Approved and Current	Major revision	8/10/2021	8/11/2021	Indefinite

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Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel Library Prep Procedure

Oregon State Public Health Laboratory

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I. PURPOSE:

A. The purpose of this procedure is to utilize the Ion AmpliSeq™ SARS-COV-2 Insight Research Panel Assay as a targeted NGS solution to sequence and analyze the entire SARS-CoV-2 genome of positive SARS-CoV-2 (SC2) specimens received at the OSPHL. It provides an efficient, high-throughput end-to-end workflow for monitoring genomic evolution, which is critical during a rapidly developing pandemic.

B. The Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel is a comprehensive next-generation sequencing (NGS) assay used to prepare Ion AmpliSeq™ libraries from SARS-CoV-2 samples using 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. Sequencing of the libraries follows on an Ion GeneStudio™ S5 Plus System with >99% coverage of the SARS-CoV-2 genome (~30 kb) and covers all potential strains. Sequencing results are then analyzed the with Torrent Suite™ Software and the sequence is then used to identify and annotate variants and specific amino acid substitutions with public or private databases to perform multi-sample comparisons.

II. SCOPE / SPECIMEN ACCEPTANCE CRITERIA:

A. A sample containing as little as 50 copies of viral RNA can be used to prepare an Ion AmpliSeq™ SARS-CoV-2 Insight Research Panel library. For optimal results, it is recommended to use a viral copy number in the 200 to 200,000 range, or an amount of *total* RNA between 1–10 ng. The amount of viral RNA among samples should be approximately equivalent so that the target amplification conditions you select are optimal for all samples.

B. OSPHL will use the Ct value of each positive SC2 specimen obtained from the CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex assay to estimate copy number of each specimen based on the table below. Specimens with a Ct value fitting the testing criteria will be sequenced.

Titer	Viral copy number	Ct Value (N protein)
Low	50-1500	25-29
Medium	1500-50000	20-25
High	50000-1500000	15-20

III. DEFINITIONS:

Ct value: The cycle number when the fluorescence of a PCR product can be detected above the background signal.
Library: A collection of similarly sized DNA fragments with known adapter sequences added to the 5' and 3' ends.

IV. ROLES AND RESPONSIBILITIES:

It is the responsibility of the laboratorian (microbiologist 2 and 3) to ensure that:

- All lab policies are followed, and sterile techniques are enforced.
- All laboratory equipment is calibrated and maintained consistent with manufacturer's recommendations.
- All reagents have been stored adequately and are not used past their expiration date.

V. EQUIPMENT / SUPPLIES / REAGENTS:

REAGENTS	
RNA Reverse transcription:	
Ion Torrent™ NGS Reverse Transcription Kit	Cat. # A45003
Library Preparation:	
Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay	Cat. # A51305
Amplicon Digestion:	
Ion AmpliSeq™ Library Kit Plus (FuPa)	Cat. # 4488990
Barcode Adapters:	
Ion Xpress™ Barcode Adapters 1–96 Kit	Cat. # 4474517
IonCode™ Adapters	Cat. # A29751
Amplicon/Library Purification:	
Agencourt™ AMPure™ XP	Cat. # A63881
Absolute Ethanol, 200 proof, Molecular Biology Grade	Cat. # T038181000
UltraPure™ DNase/RNase-Free Distilled Water	Cat. # 10977015
Library Quantification:	
Qubit™ Fluorometry 4.0	Cat. # Q33238
Qubit™ 1X dsDNA HS Assay Kit	Cat. # Q33230 or Q33231
EQUIPMENT	
PCR Cabinet – NuAire, model 126-300	Cat. # A35422
SimpliAmp™ Thermal Cycler	Cat. # A35422
Eppendorf® Centrifuge 5430/5430R	Cat. # EP022620645
myFuge 12 Micro-Centrifuge	Cat. # 50-550-338
Vortex mixer	
Rainin Pipettors – 2µl, 20µl, 200µl and 1000µl	
Rainin Multichannel Pipettors – 20µl and 200µl	
Eppendorf™ PCR Cooler	Cat. # 05-403-00
DynaMag™ - 96 Side Magnet	Cat. # 12331D
Qubit® 4.0 Fluorometer	
SUPPLIES	
MicroAmp™ Optical 96-Well Reaction Plate	Cat. # N8010560
MicroAmp™ Optical 8-Cap Strips	Cat. # 4323032
MicroAmp™ 8-Cap Strip, clear	Cat. # N8010535
Thermo Scientific Reagent reservoirs – 25ml	Cat. # 95128095
Low Protein Binding Collection Tubes (1.5 ml)	Cat. # 90410

Rainin 20µl filter tips, Terra rack	Cat. # 17014961
Rainin 200µl filter tips, Terra rack	Cat. # 17014963
Rainin 1000µl filter tips, Terra rack	Cat. # 17014967
RNase Away™	Fisher Scientific #21-236-21 or equiv.
Coverage Spray HB Plus	Steris #1624-77 or VWR #4212-963

VI. HAZARDOUS SUBSTANCES / SAFETY PRECAUTIONS: BIOHAZARD

A. Lab coats and gloves must be worn when performing this procedure.

VII. HAZARDOUS CHEMICAL WASTE DISPOSAL:

A. The IonChef deposits chemical waste into a primary container located in the instrument. After each run, the primary waste container emptied into a secondary waste container prior to disposal.
B. Chemical waste from the secondary container must be disposed of by Metro. Please refer to the Hazardous Chemical Waste Disposal procedure for more information about this process.
C. Ethanol waste can be disposed of in the lab sink under running water. Ensure that gloves, gowns are worn whenever using ethanol. Eye protection may be used as well.

VIII. INSTRUMENT START-UP / MAINTENANCE:

A. SimpliAmp™ Thermal Cycler

1. After each use
    a. Clean the housing or external part of the thermal cycler with a soft cleaning cloth or paper with approved disinfectant.
2. Annual QC Check
    a. Annual QC will be done through the “self-verification test feature” on the instrument. The check includes testing the block, heated cover, and other components.
3. As needed
    a. The thermal area lid should be cleaned with a cloth or soft cleaning paper moistened with isopropyl alcohol.
    b. Clean the wells with water followed by Isopropanol

B. Qubit® 4.0 Fluorometer

1. The Qubit® 4.0 Fluorometer does not need regular maintenance.
    a. Do not perform any repairs or service on the Qubit® 4.0 Fluorometer to avoid damaging the instrument.
    b. Do not expose the Qubit® 4.0 Fluorometer to direct sunlight.
    c. Clean the surface of the Qubit® 4.0 Fluorometer with a damp cloth.
    d. To clean the touch screen, turn off the Qubit® 4.0 Fluorometer, disconnect the power cable, and clean the touch screen with a soft cloth lightly moistened with

LCD cleansing detergent.

IX. CALIBRATION:

A. Thermocycler

For further information on the thermocycler system calibration and temperature maintenance procedures, refer to SimpliAmp™ Thermal Cycler User Guide (Pub. No. MAN0009889)

X. QUALITY CONTROL:

A. Quantification with Qubit 4.0

1. Samples with Qubit readings of >1 ng/ml will be accepted for sequencing.
2. Samples with Qubit readings less < 1 ng/ml or "too low" may be pooled undiluted and re-processed if initial Ct values follow appropriate testing criteria and fall below Ct 28.

XI. PROCEDURE:

A. Sequencing worksheet setup

1. Create a run plan in a 96-well format using an Excel spreadsheet containing the specimens to be sequenced. A negative template control (NTC) and a positive SC2 sequencing control should be included in every run.

2. Fill out the Ion S5 Sequencing Run Worksheet with run name, specimen ID, qPCR Ct values, and control lot numbers. This will be used during the library quantification and dilution step in Section K.

B. Isolate and quantify viral nucleic acid (NA)

1. Use the Ct result from the CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex assay to estimate copy number in your specimen. Determine the copy number of the specimen according to the known copy number in control reactions. See table below.

Titer	Viral copy number	Ct Value (N protein)
Low	50-1500	25-29
Medium	1500-50000	20-25
High	50000-1500000	15-20

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

The Ion Torrent™ NGS Reverse Transcription Kit will be used to reverse transcribe SARS-CoV-2 RNA for manual library preparation.

***Note:** Preparation of the NGS Reverse Transcription master mix will occur in the Virology PCR Clean room (1601).*

1. Thaw the NGS 5X Reaction mix to room temperature before use and then vortex to mix. Centrifuge briefly before use.

2. Combine the following components to make a master mix for each reaction. (Allow for pipetting loss by adding approximately 10% overage or ~2 reactions.)

NOTE: Use the Ion S5 RT and cDNA Synthesis Setup Worksheet to calculate the required amount of master mix.

Component	**Volume**
Ion Torrent™ NGS 5X Reaction Mix	2 µl
Ion Torrent™ NGS 10X Enzyme Mix	1 µl
Total volume per sample	**3 µl**

3. Place 96-well PCR plate on an Eppendorf® PCR cooler (stored at -20°C).

4. Aliquot **3 µl** of NGS Reverse Transcription master mix into each sample well of a 96-well PCR plate according to plate map.

5. Cover the plate and move the PCR plate containing master mix from the PCR Clean Room to the S5 Sequencing processing bench for sample addition.

4. Add **7 µl** of sample to the appropriate sample well.

5. Seal the plate with MicroAmp™ 8-cap strips, vortex the plate, and briefly centrifuge to collect droplets.

6. Load the plate into the SimpliAmp™ thermocycler, then run the "Reverse Transcription" method with the following parameters:

REPS	1	1	1	1
TIME	10:00	10:00	5:00	Hold
TEMP	25°C	50°C	85°C	10°C

⬣ STOPPING POINT: Samples can be stored at 10°C for up to 16 hours on the thermocycler. For longer term, store at −20°C.

D. Prepare cDNA target amplification reactions

1. Combine the following components to prepare the amplification master mix for each reaction. (Allow for pipetting loss by adding ~2 reactions per 8 samples.) Aliquot mix into an 8-tube strip for easy multi-channel transfer into specimen reaction wells.

NOTE: Use the Ion S5 RT and cDNA Synthesis Setup Worksheet to calculate the required amount of master mix. *This can be made at the same time as the NGS Reverse Transcription master mix and stored at 4^{o}C until use.*

Component	**Volume**
5X Ion AmpliSeq™ HiFi Mix	4.5 µl
Nuclease-free Water	3.5 µl
Total volume per sample	**8.0 µl**

2. Place 2 clean 96-well PCR plates on Eppendorf® PCR coolers and label them **Primer Pool 1** and **Primer Pool 2**.

3. Using a repeater pipettor, add **2 µl** of each primer pool into the appropriate sample well of each plate and set aside.

4. Remove the reverse transcription plate from the thermocycler and centrifuge.

5. Remove the 8-cap strips and add **8 µl** of the master mix to the entire volume of the reverse transcription reaction. **Total volume = 18 µl.**

6. Mix thoroughly by pipetting up and down 5 times, then transfer **8 µl** of each sample into the 96-well PCR plates containing the separate primer pools. **Total volume = 10 µl per well.**

7. Seal the plates with 8-cap strips, vortex, and briefly centrifuge.

8. Load the plate into the SimpliAmp™ thermocyclers, then run the "cDNA Target Amplification" method with the following parameters:

REPS	1	20x		Hold
TIME	2:00	0:15	4:00	∞
TEMP	98^{o}C	98^{o}C	60^{o}C	10^{o}C

NOTE: OSPHL has optimized the required cycles based on the guidelines below and the quality of samples received for sequencing.

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.

Titer	Viral copy number	# of amplification cycles
Low	50-1500	26
Medium	1500-50000	20
High	50000-1500000	15

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

E. Combine target amplification reactions

1. Place a clean 96-well PCR plate on an Eppendorf® PCR cooler and using the repeater pipettor add **2 µl** of FuPa Reagent to each sample well. Set aside.

2. Remove plate from the thermocycler and centrifuge briefly to collect the contents at the bottom of the wells.

3. Place the plates on an Eppendorf® PCR coolers.

4. Carefully remove the 8-cap strips to prevent any splashing.

F. Partial digestion of amplicons

1. Combine each 10-µL target amplification reaction into the single well containing FuPa Reagent. **Total volume per sample = 22 µl.**

NOTE: Perform this step on ice or a cold block, then quickly proceed to incubation. ***FuPa Reagent is viscous. Pipet slowly and mix thoroughly.***

2. Mix by pipetting at least half the total volume up and down at least 5 times.

3. Use the 8-cap strips to seal the wells and centrifuge briefly.

4. Load the plate into the SimpliAmp™ thermocycler, then run the “Amplicon Digestion” method with the following parameters:

REPS	1	1	1	Hold
TIME	10:00	10:00	20:00	60:00
TEMP	50°C	55°C	60°C	10°C

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

G. Ligation of adapters to the amplicons

1. IonCode™ Adapters are provided at the appropriate concentration and include forward and reverse adapters in a single well. No further handling is necessary. IonCode™ Adapters will be used in the following steps. When Ion Xpress™ adapters need to be used, follow the guidelines in the box below for preparation instructions.

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

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

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

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

[1] X = barcode chosen

Note: Store diluted adapters at –20°C.

1. Thaw the Switch Solution. *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. Remove the plate from the thermocycler and briefly centrifuge the plate to collect the contents.

3. Carefully remove the 8-cap strips then add the following components in the order listed to each well containing the digested amplicons.

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

4. Order of addition:

Component	**Volume**
Switch Solution	4 µl
IonCode™ Adapters (or)	
diluted Ion Xpress™ barcode adapter mix (for barcoded libraries)	2 µl
DNA Ligase	2 µl
Total volume (including ~22 µl of digested amplicon)	**~30 µl**

5. Mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate with 8-cap strips. You may also vortex the sealed plate.

5. Centrifuge briefly and load the plate into the SimpliAmp™ thermocycler, then run the "Amplicon Ligation" method with the following parameters:

REPS	1	1	1	Hold
TIME	30:00	5:00	5:00	∞
TEMP	22°C	68°C	72°C	10°C

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

H. Purify ligated amplicons with adapters and barcodes

1. Bring the Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly and aliquot into a smaller aliquot for single day use.

2. Prepare fresh 70% ethanol for use in the following steps.

3. Remove the plate from the thermocycler and centrifuge to collect the contents in the bottom of the wells.

4. Carefully remove the 8 cap strips then add **45 µl** (1.5X sample volume) of Agencourt™ AMPure™ XP Reagent to each library.

5. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.

6. Visually inspect each well to ensure that the mixture is homogeneous.

7. Incubate the mixture for 5 minutes at room temperature.

8. Place the plate on the DynaMag™ - 96 Side Magnet and incubate for 2 minutes or until solution clears.

9. Carefully remove and discard the supernatant without disturbing the pellet.

10. Add **150 µl** of the freshly prepared 70% ethanol, then move the plate side-to-side several times in the two positions of the magnet to wash the beads.

11. Carefully remove and discard the supernatant without disturbing the pellet.

12. Repeat steps 10 and 11 for a second wash.

13. Ensure that all ethanol droplets are removed from the wells.

14. Keeping the plate on the magnet, air-dry the beads at room temperature for 1−5 minutes. **Do not over dry.**

IMPORTANT! Residual ethanol drops inhibit library amplification. If needed, centrifuge the plate and remove remaining ethanol before air-drying the beads. Under conditions of low relative humidity, the beads air-dry rapidly. Do not over dry.

I. Library amplification

1. Prepare a master mix containing 50 µl of 1X Library Amp Mix and 2 µl of 25X Library Amp Primers for each sample. Account for pipetting loss by adding approximately 10% additional reactions.

Component	Volume
1X Library Amp Mix	50 µl
25X Library Amp Primers	2 µl
Total volume per sample	**52 µl**

2. Remove plate from the magnet.

3. Add **52 µl** of the master mix to each sample.

4. Mix by pipetting at least half the total volume up and down at least 5 times or until bead go back into solution.

5. 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 96-well plate without disturbing the pellet.

6. Use the 8-cap strips to seal the wells and centrifuge briefly.

7. Load the plate into the SimpliAmp™ thermocycler, then run the "Library Amplification" method with the following parameters:

REPS	1	5x		Hold
TIME	2:00	0:15	1:00	∞
TEMP	98°C	98°C	64°C	10°C

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

J. Amplified library purification

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

2. **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.**

    a. Remove the plate from the thermocycler and centrifuge briefly. Remove 8 cap strips.

    b. Add **25 µl** (0.5X sample volume) of Agencourt™ AMPure™ XP Reagent to each plate well containing ~50 µl of sample.

    c. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.

    d. Incubate the mixture for 5 minutes at room temperature.

    e. Place the plate on the DynaMag™ - 96 Side Magnet for at least 5 minutes or until the solution is clear.

    f. Carefully transfer the supernatant from each well to a new 96-well PCR plate without disturbing the pellet. ~75 µl

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

3. **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.**

    a. Add **60 µl** (1.2X original sample volume) of Agencourt™ AMPure™ XP Reagent to the supernatant.

    b. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.

    c. Incubate the mixture for 5 minutes at room temperature.

    d. Place the plate on the magnet for 3 minutes or until the solution is clear.

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

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

f. Add **150 µl** of freshly prepared 70% ethanol to each well, then move the plate side to side several times in the magnet to wash the beads.

g. Carefully remove and discard the supernatant without disturbing the pellet.

h. Repeat steps **f** and **g** for a second wash.

i. Ensure that all ethanol droplets are removed from the wells.

j. Keeping the plate in the magnet, air-dry the beads at room temperature for 2−5 minutes. **Do not over dry.**

k. Remove the plate from the magnet.

l. Add **50 µl** of Low TE to the pellet to disperse the beads.

m. Mix by pipetting at least half the total volume up and down at least 5 times.

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

o. Place the plate in the magnet for at least 2 minutes or until solution is clear, then transfer supernatant to a new 96-well plate without disturbing the beads.

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

K. Library quantification and dilution factor calculation

1. Analyze 10 µl of each amplified library using the Qubit™ 4.0 Fluorometer and the Qubit™ dsDNA HS Assay Kit.

2. 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*.

3. Determine the amplified library concentration using Qubit™ 4.0 Fluorometer:

   a. Prepare the Qubit™ standards as directed in the user guide (**10 µl** standard + **190 µl** Qubit™ dsDNA HS Buffer), mix well, and incubate for at least 2 minutes.

   b. For each sample, combine **10 µl** of the amplified Ion AmpliSeq™ library with **190 µl** of Qubit™ dsDNA HS Buffer, mix well, and incubate for at least 2 minutes.

   c. On the Qubit™ 4.0 Fluorometer home screen, select dsDNA then 1x dsDNA High Sensitivity.

   d. Measure the prepared standards.

e. Select the sample concentration as "ng/ml" with an input value of 10 µl.

f. Measure the library concentration for each sample and write down the concentrations for each on the printed Ion S5 Sequencing Run Worksheet.

4. Enter the library concentrations into the Ion S5 Sequencing Run Worksheet to determine the dilution for ~100pM (20ng/ml) concentration. If concentration is over 1000 ng/ml, the library will be diluted 1:10 using low TE and normalization will be calculated from that value. **Dilution values will be calculated automatically.**

Note: *Expected amplicon size is ~300 bp (avg. 225 bp with 80 bp adapters = ~300bp), 100pM solution will contain ~20ng/ml DNA.*

5. Dilute/normalize all libraries to ~100 pM (20ng/ml) with Low TE.
6. For each library pool, combine **5 µl** of each normalized library, mix, and centrifuge.
7. Seal the 96-well plate with the undiluted libraries with 8-cap strips and store at 4°C.
8. Proceed to Ion Chef template preparation.

Note: Libraries can be stored at 4–8°C for up to 1 month. For longer, store at –10°C to –30°C.

XII. QC ACCEPTANCE CRITERIA:

A. Library quantification with Qubit reading within 1 and 1000 ng/ml will be accepted and follow appropriate dilution for normalization. Any reading above 1000 ng/ml will be diluted 1:10 and normalized from this dilution. Readings of "too low" will be processed undiluted or removed from run, when necessary. If the Ct value of the original specimen is <28, the specimen will be reprocessed and sequencing repeated.

XIII. TROUBLESHOOTING:

Observation	**Possible cause**	**Recommended action**
Qubit reading "Too High"	Higher viral copy number	Make a 1:2 (1:4) dilution of the library and re-take qubit measurement. Multiply the value x2 (x4) for copy number.
Qubit reading "Too Low"	Low viral copy number	Confirm Ct value for sample and contact supervisor.

XIV. REFERENCES:

ThermoFisher Scientific, the *Qubit™ dsDNA HS Assay Kits User Guide* (Pub. No. MAN0002326).

ThermoFisher Scientific, *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003)

ThermoFisher Scientific, SimpliAmp Thermal Cycler User Guide (Pub. No. MAN0009889)

Ion AmpliSeq™ SARS-CoV-2 Insight Research Assay Product Information Sheet (Pub. No. MAN0024917 B.0)