Mar 13, 2023

Targeted NGS feline respiratory panel including SARS-CoV-2 for Ion Torrent platform

DOI
dx.doi.org/10.17504/protocols.io.j8nlkk75wl5r/v1

Jobin J Kattoor[1], Rebecca P Wilkes[1]

[1]Indiana Animal Disease Diagnostic Laboratory at Purdue University

Vet LIRN

Rebecca P Wilkes
Indiana Animal Disease Diagnostic Laboratory at Purdue Unive...

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.j8nlkk75wl5r/v1

Protocol Citation: Jobin J Kattoor, Rebecca P Wilkes 2023. Targeted NGS feline respiratory panel including SARS-CoV-2 for Ion Torrent platform. **protocols.io https://dx.doi.org/10.17504/protocols.io.j8nlkk75wl5r/v1**

Protocol status: Working
We use this protocol and it's working

Created: August 12, 2022

Last Modified: March 13, 2023

Protocol Integer ID: 68573

Keywords: ngs of feline respiratory pathogen panel, ngs feline respiratory panel, feline respiratory pathogen panel, feline respiratory panel, targeted ng, including sar, sar, ng, ion torrent platform

Funders Acknowledgements:
Vet-LIRN
Grant ID: 1 U18 FD007495

Disclaimer

Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the Food and Drug Administration.

Abstract

The current procedure is for NGS of feline respiratory pathogen panel including SARS-CoV-2 for Ion Torrent platform. Note, sensitivity of the method greatly depends on type and quality of samples.

Guidelines

Prepare nucleic acid extraction in a clean extraction room to prevent contamination.

Materials

Equipment:

- Biosafety cabinet
- PCR cabinet
- Vortex mixer
- Microcentrifuge with rotor for 1.5 mL or 2 mL tubes, capable of attaining 17,900 x g
- Bench top centrifuge
- Magnetic Particle processor (KingFisher Flex, MagMAX-Express 96, Biosprint 96 or equivalent)
- Ion Chef™ Instrument
- Ion GeneStudio™ S5 System
- Thermocycler
- Qubit Fluorometer
- DynaMag-96 Side Magnet (or similar)/DynaMag-2 (or similar)
- Geneious Prime (**https://www.geneious.com/**) or similar for sequence evaluation

Supplies:

- MagMAX Core extraction reagents
- DMEM
- 5.0 mL screw cap tubes
- NGS Reverse Transcription Kit
- Ion AmpliSeq Library Kit Plus (Manual library prep) or Ion AmpliSeq™ Kit for Chef DL8 (Automated library prep)
- WGAG19041_PRD_CFPv01 CFP_with_SARS_2 pool 1 and WGAG19041_PRD_CFPv01 CFP_with_SARS_2 pool 2 (Primer pools are available from ThermoFisher Scientific under number PURDUE_CANINE_FELINE_COMBO2.20211215)
- Ion Xpress Barcode Adapters for multiplexing (Manual library prep) or IonCode™ Barcode Adaptors 1-384 Kit (Automated library prep) (ThermoFisher Scientific)
- Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef (ThermoFisher Scientific)
- Ion S5 sequencing supplies (ThermoFisher Scientific)
- AmPure XP reagent (Beckman Coulter)
- Qubit dsDNA HS Assay Kit (ThermoFisher Scientific)
- Gloves
- Lab coat
- Ethanol (70%)
- Low TE
- Microcentrifuge tubes (0.1 mL 8 strip tubes or 0.1 mL or 0.25 mL, 0.5mL and 1.5 mL)
- Pipets and pipet tips

Troubleshooting

Before start

Prior to chip loading and sequencing:

Upload the reference and bed files provided below into the Ion S5 Torrent Server under Reference Sequences (.FASTA) and target files (.BED) using Import Custom Reference.

Nucleic acid extraction

1 **KingFisher Flex MagMAX Core extraction protocol**
In the extraction room, prepare the sample as follows.

0.2 Add 2 mL of DMEM to 5 mL tube.

0.3 Swirl the anterior nasal or throat swab and break off the swab tip.

0.4 Vortex vigorously for 00:02:00 or until the sample is suspended. 2m

0.5 Use 200 µL of supernatant for the downstream process.

1 **Prepare the Lysis/Binding solution.**
In the clean room, combine the following components for the required number of samples.

	A	B
	Component	**Volume per sample**
	MagMAX Core Lysis solution	350µL
	MagMAX Core Binding solution	350µL
	Total Lysis/Binding solution*	700µL

**Later used in step 4.4. in the extraction room*

1.1 Vortex at maximum speed for 00:00:10 . 10s

1.2 Store at room temperature for up to 24:00:00 1d

2 **Prepare the Bead/ Proteinase K mix.**
In the clean room, prepare the following to be added to the lysis plate.

2.1 Vortex the MagMAX Core Magnetic Beads thoroughly to ensure the beads are fully resuspended

	A	B
	Component	**Volume per sample**
	MagMAX Core Magnetic Beads	20µL
	MagMAX Core Proteinase K	10µL
	Total bead mix	**30µL**

2.2 The bead/ Proteinase K mix can be stored at 4°C for up to one week. Proceed to the next step.

3 Prepare the sample plates.
In the clean room, grab four deep well plates and prepare them as follows.

	Plate position	**Plate ID**	**Reagent**	**Volume per well**
	1	Lysis Plate	Bead mix	30µL
	2	Wash Plate	MagMAX Core Wash Solution 1	500µL
	3	Wash Plate 2	MagMAX Core Wash Solution 2	500µL

4	Elution Plate	MagMAX Core Elution Buffer	90µL

3.1 Take the prepared plates to an extraction hood in the extraction room and add 200 µL of each sample in the Lysis Plate.

3.2 Mix the sample with the Bead mix by pipetting up and down several times. Let the mixture incubate for 2 minutes.

3.3 Add 700 µL of Lysis/Binding solution (from step 2) to each sample in the first plate (Lysis plate).

3.4 Turn the power on to the magnetic particle processor

3.5 Using the up and down arrow key, select the program MagMAX Core Flex.

3.6 Press the start key. When prompted, load the appropriate plate on the instrument. Properly orient the plates

3.7 Press the start key to advance the carousel to the next plate position to load.

3.8 When the final plate is loaded, place a deep well comb on the top of the first plate (Lysis Plate) and press the start key. When the program is complete, the instrument will prompt to remove elution plate. Remove the elution plate and set it in an extraction hood. Press start to advance the carousel and remove all other plates as prompted. Dispose of all the wash /lysis plates by placing in a biohazard bag.

3.9 The eluted product can be used immediately or stored in a labelled 1.5mL tube or sealed elution plate (96 well) at -10°C until use.

Reverse transcription (RT) using Ion Torrent NGS Reverse Transcription Kit 45m

4 **Reaction is set up for each sample using the following table:** 10m

	A	B
	Component	**Volume**
	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	**10µL**

Note

Depending on the number of samples 96 well plate/ 8 strip tube or individual 0.1-0.25 PCR tubes can be used for setting up the reaction.

4.1 Mix the reaction mixture well and seal the plate/close PCR tubes

4.2 Execute the following thermocycling conditions on a thermocycler. 30m

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

4.3 Short spin the PCR tube/plate to bring down the contents (cDNA) to the bottom of the tubes.

4.4 Proceed to the section **Option 1** or section **Option 2** or store at -30 °C to -10

Option 1: Library prep using Ion AmpliSeq Library Kit Plus (Manual library prep method)

7h

5 **Preparing cDNA target amplification reaction**

5.1 A master mix is prepared according to the following table

10m

	A	B
	Component	**Volume**
	5X Ion Ampliseq HiFI mix (red cap)	5 µL
	cDNA	7.5 µL
	Nuclease free water	to 12.5 µL
	Total volume	**12.5 µL**

5.2 Aliquot 5 µL of master mix into two PCR tubes.

10m

5.3 Add 5 µL of primer pool 1 to first tube and primer pool 2 to the second tube. Mix the reaction using pipette.

Note: For SARS CoV-2 two different primer pools are used (WGAG19041_PRD_CFPv01 CFP_with_SARS_2 pool 1 and WGAG19041_PRD_CFPv01 CFP_with_SARS_2pool 2)

5.4 Seal the tubes and proceed with the thermocycling shown in the following table

3h

	A	B	C
	Stage	**Temperature**	**Time**
	Hold	99°C	2 minutes
	Denature*	99°C	15 seconds
	Anneal and extend*	60°C	4 minutes
	Hold	10°C	Hold

**The cyclic condition is repeated 30 times*

6 **Combine the target amplification reaction and partial digestion of the targets**

6.1 Short spin the reaction tubes after thermocycling to collect the contents to the bottom of the wells.

6.2 Combine the 10 µL target reactions into a single tube and proceed for partial digestion using FuPa reagent. 10m

6.3 Add 2 µL of FuPa reagent to the combined targets to make the volume to 22 µL. Mix well with pipette and proceed for thermocycling as shown in the following table. 50m

	Temperature	Time
	50°C	10 minutes
	55°C	10 minutes
	60°C	20 minutes

10°C	Hold (max to 1 hour)

** Partially digested targets can be stored -20°C for longer periods*

7 **Ligate adaptors to the amplicons and purification.**

Note

When processing multiple samples, barcodes can be used to differentiate samples

7.1 Following reaction is set up for each sample 30m

A	B
Component	**Volume**
Switch solution (yellow cap)	4 µL
Ion P1 adapter	0.5 µL
IonXpress barcode*	0.5 µL
Nuclease free water	1 µL
DNA Ligase (blue cap)	2 µL
Target from the partial digestion step	22 µL
Total volume	**~30 µL**

**IonXpress barcodes are available from 001-096. Separate barcodes are used to differentiate libraries from individual samples.*

7.2 The mixture was then incubated in the following conditions. 50m

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

7.3 The contents in the tube were briefly centrifuged after the incubation to collect it to the bottom of the wells.

7.4 Add 45 µL of Agencourt™ AMPure™ XP Reagent to each tube. Pipet up and down to mix it properly. 40m

7.5 Incubate the mixture for 3-5 minutes at room temperature and keep the magnetic rack. Incubate for 2 minutes or until solution becomes clear.

7.6 Remove the supernatant and wash the pellet with 150 µL of fresh 70% ethanol.
Rotate the tubes or move the position of the tubes so that there will be maximum contact of magnetic beads with the wash solution.

7.7 Repeat the washing step one more time and air dry the beads in the tube by keeping it open at room temperature for 2-3 minutes.

7.8 Add 20-25 µL of low ionic buffer/TE buffer/ nuclease free water to the dried magnetic beads and mix it well.

7.9 Keep the mixture in room temperature for approx. 2 minutes.

7.10 After the incubation keep the tubes back to the magnetic rack.

7.11 Remove the clear solution to a new 1.5 mL microcentrifuge tube and proceed with the next steps.

8 **Quantify and equalize the library.** 30m

8.1 Quantification of the prepared libraries can be done using Qubit fluorometer according to manufacturer's protocols.

8.2 Dilute the libraries to a concentration of ~100pM (50 µL total volume for each chip to be loaded) for using Ion 510/520/530 chip loading.

Note

Example:
The barcoded library concentration is 15,000 pM.
Dilution factor = 15,000 pM/100 pM = 150
Therefore, 1 µL of barcoded library that is diluted with 149 µL of Low TE (1:150 dilution) yields a 100-pM solution.

Note

Undiluted libraries can be stored at 4-8°C for up to 1 month and for longer periods, libraries can be stored at -30°C to -10°C.

8.3 Proceed to section creating chip plan on Ion Chef

Option 2: Library prep using Ion AmpliSeq™ Kit for Chef DL8 (Automated library prep method) 8h

9 Perform the RT step as described in section 5.1 to 5.4.

9.1 Thaw all the Ion AmpliSeq™ Kit for ChefDL8 reagent (in -20°C), DL8 solution cartridge (in 4°C) and primers (WGAG19041_PRD_CFPv01 CFP_with_SARS_2 pool 1 and 2) (in -20°C) after putting the RT reaction in the thermocycler.

9.2 Make the sample plan in Torrent suite software (TSS) in **http://10.160.129.7/** after logging in to the server using credentials. Make sure to put the Ioncode PCR plate serial number and the sample numbers correctly. Preferably, the control form number should be marked. The sample numbers should be marked correct with the corresponding Ioncode barcodes. All the optional menus can be left as such in the plan. Mark the plan name with the ongoing library name (e.g.: COVID lib 51A).

9.3 Make up the volume of cDNA from step 2.4 to 15 µL before adding to the Ioncode plate in the next step.

9.4 Add 15 µL of the cDNA into the A-H (Fig 1) well in the Ioncode plates according to the plan made in the TSS.

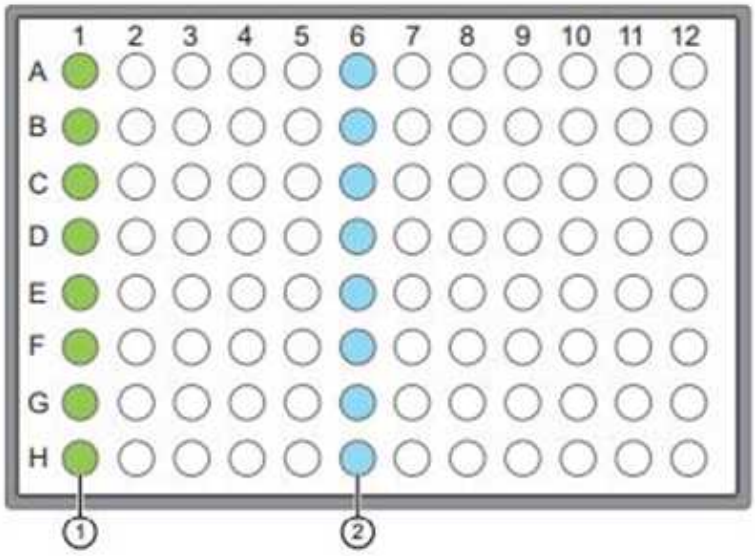


Figure 1: Plate set up for Ioncode barcode plates (ThermoFisher Scientific) for use in automatic library preparation.

Position 1(A-H): Positions for adding 15µL of cDNA.
Position 6 (A-H): Positions of lyophilized precoated Ioncode barcodes.

9.5 Add 150µL of each primer pool 1 and 2 to the first and second tubes, respectively, in the DL8 reagent cartridge. Vortex and spin down the primer pairs before placing them on the cartridge.

9.6 Take out the DL8 chef supplies.

9.7 In the Ionchef, go to set up a Run> Prepare library> and Open the chef door. Keep all the chef supplies and solution/reagent cartridges in the correct position, as shown in Figure 2. Close the door of the Ion Chef and start with the deck check.

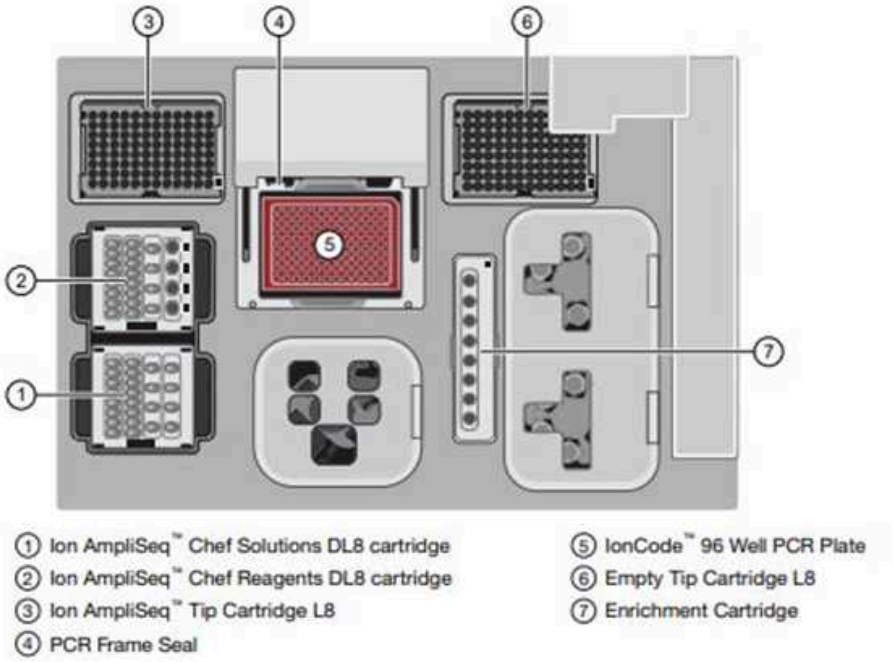


Figure 2: Positions of supplies and reagents in the Ion Chef for library prep using Ion AmpliSeq™ Kit for Chef DL8

Note

Remember to remove and save the screw caps on the tubes in the reagent cartridge.

9.8 After the deck check, see for the server name and the saved sample plan name (e.g.: COVID lib 51 A). if the plan name is not coming on the display, refresh the screen. It may go up to 2-3 refresh.

9.9 Check the prompted boxes showing number of primer (2), number of cycle (27) and extension time (4 min) if shown correct.

9.10 The program is set, and the chef will start in under 1 minute time

9.11 The program will generally go for above 7 hours according to the length and number of the cycle.

Note

After the completion of the library prep, open the door and remove the 4th tube (towards the red dot on the reagent cartridge) and close with a cap from the saved caps and mark the library name. Store the library immediately in -80°C till the chip loading step.

Take out all the consumables from the Ion Chef and go for clean the instrument.

Note

Remember to change the position of the used pipette box from the left-hand slot to the right-hand slot)

9.12 After completion of library prep, 700µL of pooled library at 100pM is obtained in the 4th tube in the reagent cartridge.

9.13 go to step #9 according to the number of samples

Note

Approximately 32 samples can be multiplexed on a single Ion 530 chip and one DL8 library prep will produce pooled library from 8 barcoded samples at 100pM concentration.

A total of 50 µL from step 10.12 is used to load the Ion Chip in the chip loading steps, and equal volumes of multiple barcoded libraries are used to load the Ion chip in the following steps (25 µL each if two sets of libraries are mixed together in a single chip)

9.14 Proceed to section creating chip plan on Ion Chef

Creating chip plan on Ion Chef

20m

10 Create a chip plan on Ion Chef

10.1 Sign-in into the TSS using credentials (**http://10.160.129.7/**).

10.2 Keep the Ion 510, 520, & 530 Reagent cartridge stored at -20ºC at room temperature for at least 30 minutes.

10.3 In the Plan tab, click on Plan New Run.Select Ion Reporter Account as "None" and Sample Grouping as "Other" and click on Next.

10.4 Click on "DNA" in the Research Application and AmpliSeq DNA in Target Technique and click on Next.

10.5 Set the instrument type (e.g., Ion GeneStudio^{TM} S5 System), Library kit (e.g.: Ion AmpliSeq Library kit plus) used accordingly and select Ion Chef.

10.6 In the Template Kit, select Ion 510, Ion 520 & Ion 530 Kit-Chef, and in Sequencing Kit, select Ion S5 Sequencing Kit from the drop-down menu.

10.7 In the Chip Type drop-down menu, select the chip accordingly to the number of different samples/barcodes used.

10.8 Barcode Set can be selected optionally depending on the type of barcode used

10.9 In the Flow tab, fill in 500 and click on **Next**.

10.10 Select the previously uploaded Plugins for analysis, including Assembler SPAdes, and File exporter, and click **Next**.

10.11 Optionally, for saving results under a specific project, a project name can be created and selected in the Projects window or click on **Next**.

10.12 Fill in the Run Plan Name and already uploaded Reference Library- canine_feline_combo_with_SARS_2 and Target region- WGAG19041_PRD_CFPv01_edit.bed from the drop-down menu.

10.13 Fill in the required Number of barcodes and Chip Barcode (9-digit alphanumeric code).

10.14 Fill in the corresponding Barcode and Sample Name in the following table and click on Plan Run.

10.15 Repeat step 11.3. to create a plan for the second chip.

10.16 After creating plans for one or both the chips, proceed to section 12.

Chip loading on Ion Chef

40m

11 Load chip on Ion Chef

11.1 At least 00:30:00 after keeping the Ion 510, 520, & 530 Reagent cartridge at room temperature, proceed with the following steps

30m

11.2 Add the diluted barcoded library pools from step 9.2 or step 10.12 into positions 1 and 2 of the Ion S5 Reagent cartridge. Position 1 of the Ion 510, 520, & 530 Chef Reagents cartridge corresponds to the libraries planned in Chip 1, and position 2 corresponds to those samples planned in Chip 2. (Figure. 3).

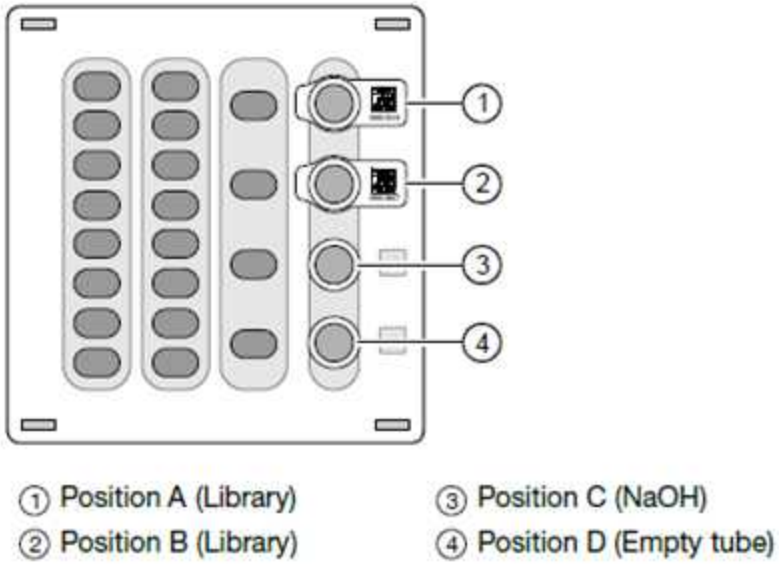
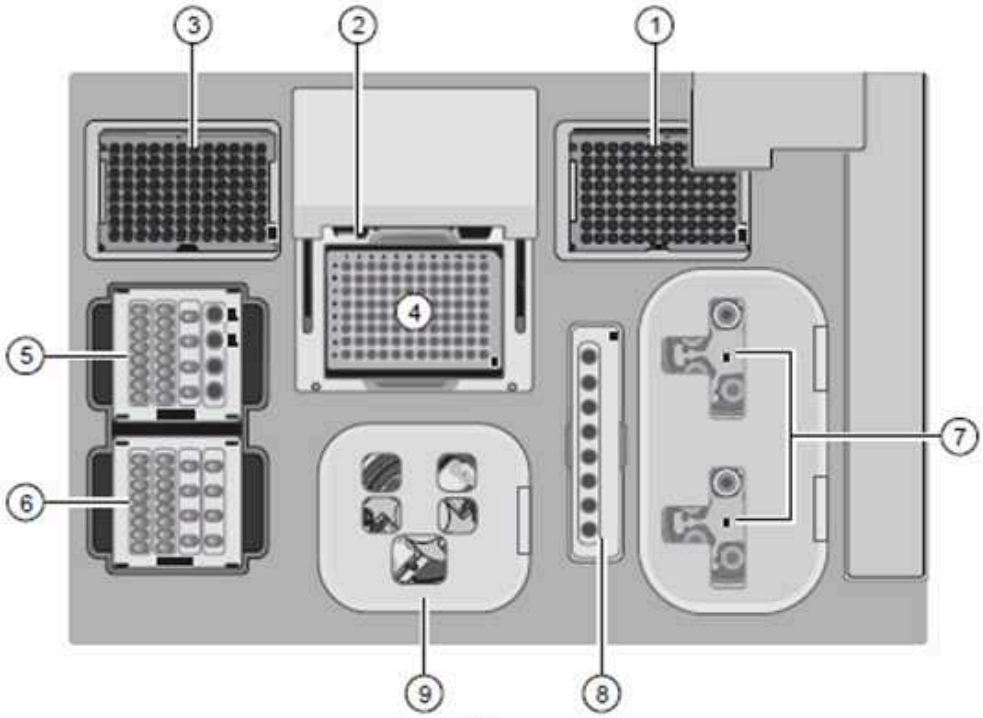


Figure 3: Position of sample and reagent tubes in Ion 510, 520, & 530 reagent cartridge. 50µL of pooled barcoded libraries are added into position A and B of the cartridge.

11.3 Click on the open symbol on the Ion Chef screen to open the door. Keep all the reagents and consumables as shown in Figure 4 following the on-screen prompt on Ion Chef.



A schematic of a loaded Ion Chef™ Instrument

① Empty tip rack (move from new Tip Cartridge position)
② Frame Seal v2
③ New Tip Cartridge
④ PCR Plate
⑤ Ion 510™ & Ion 520™ & Ion 530™ Chef Reagents cartridge
⑥ Ion S5™ Chef Solutions cartridge
⑦ Recovery Tubes and Recovery Station Disposable Lid v2
⑧ Enrichment Cartridge v2
⑨ Chip Adapter/Chip assemblies

Figure 4: Position of consumables on Ion Chef™ for Ion chip loading

11.4 Uncap all the tubes in the Ion 510, 520, & 530 Chef Reagents cartridge.

11.5 Close the door after keeping the reagents and other consumables and proceed for **Deck Scan**.

11.6 After completing Deck Scan successfully, click **Next**and select the specific plans created in section D for each Chip; Chip 1 and Chip 2. In case of no plans are shown on the screen, click **Refresh Plans**and select the plans once they are shown. Click **Next** once the plan is selected.

11.7 On the next screen, click **Timer** and select the time to end the Chip loading function on Ion Chef. On completion, the chip will be stored in Ion Chef at 4ºC for a maximum of 24 hours. Otherwise, when the run is complete, unload the Ion Chef™ Instrument. Once taken out from the Ion Chef, and if two chips were loaded at the same time, the first chip goes directly into the sequencer for sequencing, and the second chip can be stored in a chip container **at 4ºC** until use.

11.8 After unloading the used consumables except for the empty pipette tip holder (which will be moved from position 3 to position 1 as in Figure 4) from the Ion Chef, close the door of the Ion Chef and proceed with the "**Clean instrument"** function when prompted.

Sequencing using Ion GeneStudio S5

3h

12 Sequence using Ion GeneStudio S5

12.1 Each initialization with the Ion S5 sequencing kit is suitable for sequencing two chips (two sequencing runs). The Ion **S5 sequencing cartridge** should be brought to room temperature at least **2 hours prior**, and **initialization** should start at least **1 hour before** the end of Chip loading described in step 12.7.

12.2 To start the initialization of the sequencer, click on **Initialize** on the home screen of the Ion S5 sequencer.

12.3 Remove the used Ion S5 wash solution bottle and empty the wash buffer waste tank.

12.4 Place a new Ion S5 wash solution and an Ion S5 cleaning solution in the designated positions.

12.5 Place the thawed Ion S5 Sequencing cartridge into the position in the sequencer.

12.6 Place a used chip on the chip holder, close the sequencer door, and start initialization.

12.7 Initialization will take approx. 45 minutes, and when completed, click on **Home** to open the **Run** option.

12.8 Click **Run** and proceed as prompted on the screen of the Ion S5 sequencer. Replace the chip on the sequencer with the new Chip (Chip 1) from step 8.7, close the door of the sequencer and proceed as prompted.

12.9 Click on the correct plan from the drop-down menu, uncheck the **Enable post-run clean**, and tap **Review**.

12.10 Tap **Start** if the plan is shown correctly for the selected chip.

12.11 First chip sequencing will take 2h 30 mins and keep the second chip **at room temperature** at least **30 minutes** prior to the end of first chip sequencing.

12.12 When the first run is finished, click on **Run complete** to open the dialogue window for the next run.

Analysis using Torrent suite software and Geneious prime

13 **Analysis**

13.1 Open the Torrent Suite Software (TSS) (**http://10.160.129.7/**) and in the **Data** tab, click on the plan name described in section 3.15, which was created for running the sequencing.

13.2 Aligned, trimmed files available as BAM files from the TSS can be downloaded into the local drive and can be opened in Geneious prime software (**https://www.geneious.com/**).

13.3 Based on the alignment to reference sequences in the TSS, sequence reads can be viewed in the Geneious prime software.

13.4 For additional confirmation, each sequence can be subjected to a BLAST analysis at **https://blast.ncbi.nlm.nih.gov/** for the percent identity with SARS-CoV-2 sequences.