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High Throughput Semi-Automated SARS-CoV-2 Library Preparation Protocol for Ion Torrent Sequencing using Opentrons, New England Biolabs Kit, and ARTIC Primers

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ABSTRACT

This protocol is for the preparation of libraries for 96 samples of SARS-CoV-2 using the New England Biolabs NEBNext®Fast DNA Library Prep Set for Ion Torrent™ kit, ARTIC primer pools, the Ion Express Barcodes, and sequencing through Ion Torrent. The library preparation is semi-automated with the open source Opentrons robot. The volumes, concentrations, cycling conditions, and Opentrons protocols have been optimized through a series of experiments and are currently being used at our lab on a weekly basis. The samples used for this protocol were extracted after being obtained from nasopharyngeal swabs, placed in viral transport medium, and mixed 1:1 with AVL buffer (Qiagen). It is advisable to perform a screening for the samples before starting the library preparation by RT-qPCR and select the samples that have Ct values below 30.

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GUIDELINES

General guidelines that apply to the entire preparations, unless explicitely said otherwise:

1. It is preferable to pre-screen the samples with RT-qPCR and only include the samples that have Ct values below 30
2. All the reagents, samples, and master mixes should remain on ice unless explicitly indicated otherwise
3. Mixing the reagents and samples thoroughly is crucial in every step
4. It is recommended to separate the workstations and working areas into pre-PCR and post-PCR
5. Compatibility of the plastic material should be checked before running the OT-2 protocols
6. Exact positions on the deck should be used with the OT-2 (make sure if a mold is needed for the 96-well plate or not. General rule is that the mold is always used with the temperature module and is not used in the 2 protocols where the Ampure Beads purification is done)
7. In order to check the deck design for the OT-2 protocols, reagents needed, and positions, use the following link and import the respective protocol: https://designer.opentrons.com/ then go to the "Design" tab, select "Starting Deck State", and click on the "Names and Liquids" for each labware after mousing over them
8. Calibrate the OT-2 workstations every time you load a new protocol
9. It is important to use the exact material and conditions specified since any change in reagents does not guarantee good results
10. If OT-2 is not available, then the use of multi-channel pipettes and a magnet for 96-well plates can greatly reduce the hands-on time needed for the library preparations
11. Unless otherwise indicated, the timing for each step is estimated considering the OT-2 protocols will be used. These times might vary if the steps are done manually

The labware used in the OT-2 protocols are the following:

- BioRad 96-well plates (with or without the 96-well aluminum mold, depending on the protocol)
- NEST 12-well, 15mL reagent reservoirs
- NEST 2mL screwcap Eppendorf tubes with the respective aluminum block
- Opentrons 96 filter tip rack 20µL
- Opentrons 96 filter tip rack 200µL (can substitute with BioTIX filter tips without changing the protocol)

WARNING: If any of these material were substituted with another brand, the OT-2 protocols need to be changed accordingly using the protocol designer (https://designer.opentrons.com/) since otherwise the difference in the dimensions of the labware will cause the OT-2 protocol to fail. In all cases, it is highly recommended to have a trial run using nuclease-free water for all the OT-2 protocols before using them with actual samples and reagents in order to check that all the material and labware is compatible.

DISCLAMER: The volumes, concentrations, and cycling conditions in this protocol are different from those recommended by the manufacturers. Though we have tested these conditions and found them to be adequate, we cannot guarantee the results that the manufacturers can guarantee using their preset protocols. Moreover, this protocol is to be solely used for research purposes.

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MATERIALS TEXT

The equipment needed for this protocol are:

1. P10, P20, P200, and P1000 single channel pipettes
2. P10, P20, and P200 multi-channel pipettes
3. Thermal cycler (make sure that it is compatible with the 96-well plate used with OT-2)
4. 96-well plate benchtop centrifuge
5. 96-well plate magnet (if the OT-2 with the magnetic module is not used)
6. Ion Chef™ System
7. Ion GeneStudio™ S5 System
8. Qubit Fluorometer (can be replaced with any other fluorometer)
9. Opentrons OT-2 with the following:
   - P20 GEN-2 single channel pipette (to be installed on the left side)
   - P200 GEN-2 multi-channel pippette (to be installed on the right side)
   - 24-well aluminum block
   - 96-well aluminum block
   - Magnetic module (to be installed for the protocols in which it is needed)
   - Temperature module (to be installed for the protocols in which it is needed)
   - Any PC/laptop connected to OT-2 with the Opentrons App installed

The material and reagents needed for this protocol (for each 96-well plate library preparation) are:

1. Extracted nucleic acids containing SARS-CoV-2 RNA
2. SuperScript IV VILO Master Mix (Thermo Fisher Scientific) 50 reactions x 2
3. NEBNext®Fast DNA Library Prep Set for Ion Torrent™ (New England Biolabs) 50 reactions x 1
4. hCoV-2019/nCoV-2019 Version 3 Amplicon Set Primer Pools (ARTIC) x 1 (each pool) (IDT)
5. Ion Xpress Barcode Sets from 1 to 96 (Thermo Fisher Scientific) (can be replaced with less barcodes that could be re-used **only if** the samples will be loaded on separate chips)
6. Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef that contains the Ion Chef reagents, supplies, and S5 initialization reagents (Thermo Fisher Scientific) x 2 sets
7. Ion 530™ Chip Kit (containing 8 chips) (Thermo Fisher Scientific) x 1
8. Mag-Bind Total Pure NGS magnetic beads 500mL (Omega Biotek) x 1
9. Qubit dsDNA BR (Broad Sensitivity) or HS (High Sensitivity) Assay kit and tubes (if another fluorometer is used then these should be replaced accordingly) 100 assays x 1 and 100 tubes
10. Nuclease-free water x 500mL
11. Ethanol (Molecular Biology Grade) x 500mL
12. TE buffer (provided with the library preparation kit) and/or any commercial Elution Buffer (50mL)
13. 10µL (x 6), 20µL (x 7), 200µL (x 12), and 1000µL (x 1) filtered nuclease-free tip racks
14. 20µL (x 7), and 200µL (x 12) filtered nuclease-free tip racks compatible with the Opentrons OT-2 (amounts can be substracted from the above tips if the OT-2 will be used in all the steps where it can be used)
15. 96-well plates compatible with the OT-2 system and thermal cycler, with sealing film (for storing the plates and running the thermal cycling protocols) x 8
16. 2mL eppendorf tubes compatible with OT-2 x 16
17. 12-well, 15mL reagent reservoirs compatible with OT-2 x 2
18. Reagent reservoirs for manual dispensation of the Master Mixes and reagents needed for the purification steps with a multi-channel pipette x 16

DISCLAIMER:

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

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Preparation of cDNA using VILO from Thermo Fisher 1h 45m

1 For each sample dispense 7µL of the extracted nucleic acids to the respective wells in the 96-well plate (**Plate 1**) 30m

NOTE: All the calculations from here until the end of the protocol are done for 96 samples + 4 samples to account for pipetting erros

2 Prepare the following master mix (VILO master mix): 5m

- 5X VILO Reaction Mix: 200µL
- 10X SuperScript Enzyme Mix: 100µL

NOTE: The 5X VILO Reaction Mix should be at room temperature 40 minutes before being used

3 Dispense 3µL of the VILO master mix in each well of **Plate 1** 30m

NOTE: The OT-2 can perform this step using the following protocol:
VILO Master Mix Dispensation for 96 Samples.json

4 Seal **Plate 1**, perform a quick spin and place it in the thermal cycler for cDNA synthesis with the following thermal program: 40m

- 42°C for 30 minutes
- 85°C for 5 minutes
- hold at 4°C

SAFE POINT: the cDNA samples can be stored at -20°C

Amplification of the SARS-CoV-2 genome using the Primer Pools from ARTIC 4h 35m

5 Prepare **two** Master Mixes (one for each primer pool) containing: 10m

- NEBNext Q5 Hot Start Master Mix (BLUE cap): 500µL
- Pool A **OR** B (ARTIC): 250µL

NOTE: The NEBNext Q5 Hot Start Master Mix should be at room temperature 40 minutes before being used

6 Dispense 7.5µL of **each** master mix into a new 96-well plate 40m

NOTE: The OT-2 can perform this step using the following protocol:
Pool Master Mix Dispensation for 96 Samples.json (can run the same protocol twice (once for the Master Mix of Pool A and a second time for the Master Mix of Pool B) without having to re-calibrate in-between the two runs)

7 Add 2.5µL of cDNA from **Plate 1** to the respective wells in both plates (where one plate contains the Master Mix with Pool A and the second plate contains the Master Mix for Pool B) 10m

8 Seal the 96-well plates with Pools A and B, perform a quick spin to them, and place them in the thermal cycler(s) for PCR amplification with the following program: 3h 20m

- 98°C for 1 minute
- 25 or 30* cycles of:
  - 98°C for 15 seconds

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- 65°C for 5 minutes
- hold at 4°C

*The number of cycles should be adjusted according to the original Ct values of the samples where 25 cycles are recommended for samples with Ct values between 15 and 28, and 30 cycles are recommended for samples with Ct values between > 28 (although it is not recommended to include samples with Ct values > 30)

9 Mix 10µL of the PCR product from the plate of Pool A with 10µL of the PCR product from the plate of Pool B (use of a multi-channel pipette is highly recommended) 10m

NOTE: From here on, the samples must be managed in a post-PCR area

10 Tranfer 10µL of the **mixed** amplicons to a new 96-well plate (**Plate 2**) (the remaining 10µL can be stored at -20°C) (use of a multi-channel pipette is highly recommended) 5m

SAFE POINT: the samples can be stored at -20°C

End Repair using the New England Biolabs Kit 1h 25m

11 Prepare the following End Repair Master Mix: 5m

- Nuclease-free water: 1550µL
- NEBNext End Repair Reaction Buffer (GREEN cap): 300µL
- NEBNext End Repair Enzyme Mix (GREEN cap): 150µL

12 Mix 20µL of the End Repair Master Mix with the 10µL of the mixed amplicons in **Plate 2** 45m

- **NOTE:** The OT-2 can perform this step using the following protocol: **End Repair Master Mix Dispensation for 96 Samples.json** (**IMPORTANT:** For this OT-2 protocol, the total volume of the Master Mix has to be divided into 2 tubes where each tube will have a final volume of 1000µL)

13 Seal **Plate 2**, perform a quick spin and place it in the thermal cycler for end-repair with the following program: 35m

- 25°C for 20 minutes
- 70°C for 10 minutes
- hold at 4°C

Ion Express Adaptor and Barcode Ligation 2h 55m

14 Prepare the Barcode Mixes 1h 10m

NOTE: This section considers that the barcodes 1 to 96 are being used. However, it can be adjusted to use barcodes 1 to 48 for the first 48 samples in the 96-well plate and re-use the same barcodes for the second half of the plate, as long as the samples are sequenced separately. Also, barcodes 1 to 24 can be used for four sets of 24 samples in the 96-well plates as long as the samples are to be sequenced separately.

14.1 Prepare the following P1 Adapter Master Mix: 5m

- Nuclease-free water: 400µL
- P1 adapter: 200µL

14.2 Dispense 6µL of the P1 Adapter Master Mix in a new 96-well plate 20m

NOTE: The OT-2 can perform this step using the following protocol: **Adapter P1 Master Mix Dispensation for 96 Samples.json**

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14.3 Add 2µL of each barcode to a well in the 96-well plate where each well should contain a different barcode 45m

NOTE: the plate contains Barcode Mixes for three runs. They can be stored at -20°C

15 Prepare the following Barcode Ligation Master Mix: 5m

- Nuclease-free water: 900µL
- T4 DNA Ligase Buffer for Ion Torrent (RED cap): 500µL
- *Bst* 2.0 WarmStart DNA Polymerase (RED cap): 50µL

16 Add 14.5µL of the Barcode Ligation Master Mix to each well of **Plate 2** 45m

NOTE: The OT-2 can perform this step using the following protocol:
Barcode Ligation Master Mix Dispensation for 96 Samples.json (**IMPORTANT:** For this OT-2 protocol, the total volume of the Master Mix has to be divided into 2 tubes where each tube will have a final volume of 725µL)

17 Add 2.5µL of the Barcode Mix from the Barcode-Containing 96-well plate to the respective wells of **Plate 2** (use of a multi-channel pipette is highly recommended) 10m

18 Add 3µL of the T4 DNA Ligase (RED cap) to each well of **Plate 2** 20m

NOTE: It is important to add the reagents in this specific order (Master Mix first, then barcodes, and lastly the DNA ligase)

19 Seal **Plate 2**, perform a quick spin and place it in the thermal cycler to perform ligation with the following program: 25m

- 25°C for 15 minutes
- 65°C for 5 minutes
- hold at 4°C

SAFE POINT: the samples can be stored at -20°C

First Purification with Magnetic Beads 1h 18m

20 **NOTE:** This entire section can be performed by OT-2 in 54 minutes using the following protocol:
FIRST Ampure Beads Purification for 96 Samples.json
Follow Steps 21 to 31 only if performing this section manually (use of a multi-channel pipette is highly recommended). Otherwise, load the above protocol in OT-2 so that it can perform these steps automatically.

21 Add 90µL (1.8X) of Magnetic Beads to the samples in **Plate 2** and mix thoroughly 15m

22 Incubate 5 minutes at room temperature 5m

23 Place the plate on a magnet and wait 2 minutes for the solution to clear 2m

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24 Remove and discard the supernatant 10m

25 Add 180µL freshly prepared 70% ethanol 5m

26 Gently pipette the ethanol up and down 4 times and then remove and discard it 10m

27 Leave the plate at room temperature for 2 minutes (manually remove excess ethanol without disturbing the pellet if needed) 2m

NOTE: Do not let the pellet over-dry resulting in a dark brown-black color since this would lead to irreversible binding of the DNA and inability to elute the DNA

28 Remove the plate from the magnet, add 25µL of elution buffer (or TE buffer), and mix thoroughly 15m

29 Incubate at room temperature for 2 minutes 2m

30 Place the plate on the magnet and wait for 2 minutes for the solution to clear 2m

31 Transfer 20µL of the supernatant to a new 96-well plate (**Plate 3**) 10m

SAFE POINT: the samples can be stored at -20°C

Library Amplification 1h 5m

32 Prepare the following Master Mix: 5m

- NEBNext Q5 Hot Start Master Mix (BLUE cap): 2000µL
- NEBNext DNA Library Primers (BLUE cap): 180µL

NOTE: The NEBNext Q5 Hot Start Master Mix should be at room temperature 40 minutes before being used

33 Add 20µL of this Master Mix to each sample in **Plate 3** 45m

NOTE: The OT-2 can perform this step using the following protocol:
Library Amplification Master Mix Dispensation for 96 Samples.json (**IMPORTANT:** For this OT-2 protocol, the total volume has to be divided into 2 tubes where each tube will have a final volume of 1090µL)

34 Seal **Plate 3**, perform a quick spin and place it in the thermal cycler for PCR amplification with the following program: 15m

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- 98°C for 30 seconds
- 7 cycles of:
  - 98°C for 10 seconds
  - 58°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- hold at 4°C

SAFE POINT: the samples can be stored at -20°C

Second Purification with Magnetic Beads 1h 18m

35 **NOTE:** This entire section can be performed by OT-2 in 54 minutes using the following protocol: **SECOND Ampure Beads Purification for 96 Samples.json** (if the first and the second purifications with magnetic beads are performed on the **same day**, then the same reservoir well can be re-used for the second purification).

Follow Steps 36 to 46 only if performing this section manually (use of a multi-channel pipette is highly recommended). Otherwise, load the above protocol in OT-2 so that it can perform these steps automatically.

36 Add 36µL (0.9X) of Magnetic Beads to the samples in **Plate 3** and mix thoroughly 15m

37 Incubate 5 minutes at room temperature 5m

38 Place the plate on a magnet and wait 2 minutes for the solution to clear 2m

39 Remove and discard the supernatant 10m

40 Add 180µL freshly prepared 70% ethanol 5m

41 Gently pipette the ethanol up and down 4 times and then remove and discard it 10m

42 Leave the plate at room temperature for 2 minutes (manually remove excess ethanol without disturbing the pellet if needed) 2m

NOTE: Do not let the pellet over-dry resulting in a dark brown-black color since this would lead to irreversible binding of the DNA and inability of eluting the DNA

43 Remove the plate from the magnet, add 30µL of elution buffer (or TE buffer), and mix thoroughly 15m

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44 Incubate at room temperature for 2 minutes 2m

45 Place the plate on the magnet and wait for 2 minutes for the solution to clear 2m

46 Transfer 25µL of the supernatant to a new 96-well plate (**Plate 4**) 10m

SAFE POINT: the libraries can be stored at -20°C

Library Quantification, Normalization and Sequencing 2d 5h 20m

47 Quantify the samples in the **Plate 4** using either the Broad Range (BR) or the High Sensitivity (HS) kit and Qubit Fluorometer (Thermo Fisher) or any other compatible kit and machine at your disposal 2h

NOTE: Make sure that the output is in ng/mL and that it is adjusted to the original concentration in the sample

48 Normalize the samples' concentrations to 24ng/mL (equivalent to 100pM) by mixing the calculated sample amount with elution buffer (TE) to a final volume of 100µL in a new 96-well plate (**Plate 5**) 2h 30m

NOTE: The final volume of the normalized sample can be adjusted if the sample volume to be added is too large or too small, as long as the final volume of the normalized sample is more than 10µL

49 Create the pools for sequencing mixing 10µL from each normalized sample in an eppendorf tube. Four pools of 24 samples each may be prepared from the 96 samples. 20m

NOTES:

1. According to our experience 20 to 30 samples may be included in a 530 Chip with good coverage and depth. If a larger or a smaller chip is to be used then the number of samples to be included in a single pool should be adjusted accordingly
2. Always be sure not to mix samples with the same barcode in the same pool

50 Use 25µL from each pool in order to load the Ion Chef with 530 Chips. Two pools can be simultaneously loaded in two different chips using the same Ion Chef reagents. 20m

NOTES:

1. For Steps 50 to 52, follow the manufacturer's instructions in order to load the Ion Chef and sequence with the Ion S5 system
2. The Ion Chef Reagents need to be at room temperature 45 minutes before being used
3. Change the templating protocol to 400bp while planning the run since the average amplicon length using the ARTIC primer pools is 320bp

51 Initialize the Ion S5 system using the S5 solutions 10m

NOTES:

1. The Ion S5 Reagents need to be at room temperature 45 minutes before being used
2. One initialization can be used to sequence two 530 Chips if the number of flows is selected to be 550 (without the need to wash in-between the two chips)
3. Once the initialization is complete, both chips have to be sequenced within 24 hours

52 Sequence the chips one after the other using the Ion S5 system 2d

NOTES:

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1. On day 1, load the Ion Chef with two pools and initialize the S5 in the afternoon
2. On day 2, load the first chip in the morning and then directly load the second chip after it (keep the second chip in the membrane protector at 4°C and remove it to room temperature 45 minutes before sequencing)
3. On day 2, load the Ion Chef with the other two pools and re-initialize the S5 in the afternoon after it has finished sequencing the first 2 chips
4. On day 3, load the first chip in the morning and then directly load the second chip after it (keep the second chip in the membrane protector at 4°C and remove it to room temperature 45 minutes before sequencing)
5. Keep the Ion S5 system on after it finishes sequencing and until it processes all the data.

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