**nCoV-2019 sequencing protocol  
\*Adapted from** [**Josh Quick**](https://www.protocols.io/researchers/josh-quick) **(University of Birmingham) for Diaz-Munoz Lab**

**MATERIALS:**

* Primers 25nm, desalted, ideally LabReady formulation from ***IDT nCoV-2019/***[***V3***](https://github.com/artic-network/artic-ncov2019/tree/master/primer_schemes/nCoV-2019/V3)
* Extraction kits; Zymo Quick-RNA Viral Kit ***Zymo R1034*** **or**
* QIAamp Viral RNA Mini ***Qiagen 52904***
* SuperScript IV (50 rxn) ***Thermo 18090050***
* dNTP mix (10 mM each) ***Thermo R0192***
* Random Hexamers (50 µM) ***Thermo N8080127***
* RNase OUT (125 rxn) ***Thermo 10777019***
* Q5 Hot Start HF Polymerase ***NEB M0493S***
* NEBNext Μltra II End-prep ***NEB E7546S***
* NEBNext Quick Ligation Module ***NEB E6056S***
* Native Barcoding Expansion Kit 1-12 ***Nanopore EXP-NBD104***
* Native Barcoding Expansion Kit 13-24 ***Nanopore EXP-NBD114***
* Sequencing Auxiliary Vials ***Nanopore EXP-AUX001***
* Short Fragment Buffer Expansion kit ***Nanopore EXP-SFB001***
* Flow Cell Priming Kit ***Nanopore EXP-FLP002***
* R9.4.1 flow cells ***Nanopore FLO-MIN106***
* Strip tubes

**PROCEDURE:**

* **cDNA preparation *(~3 hours)***
  + In flu room (Briggs 257)
    - Don PPE and wipe down biosafety cabinet/counters with 10% bleach
    - Set PCR machine to hold @ 65ºC  
      **A picture containing indoor, table, desk, computer

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    - Get 1.5ml microcentrifuge (microcentrifuge tubes) and strip tubes from the small black cabinet near the hood (if not available, the main lab has in the shelves above post-PCR area)
    - Get an insulated cool tube rack and PCR rack from the freezer (may have to find one in main lab)
    - Make master mix I in microcentrifuge tubes

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|  | **Component** | **Volume** |
|  | 50μM random hexamers | 1μl |
|  | 10mM dNTPs (10mM each) | 1μl |
|  | Template RNA | 11μl |
|  | *Total* | *13μl* |

* + - Make master mix II in microcentrifuge tubes to add to annealed template RNA

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|  | **Component** | **Volume** |
|  | SSIV buffer | 4μl |
|  | 100mM DTT | 1μl |
|  | RNaseOUT RNase Inhibitor | 1μl |
|  | SSIV Reverse Transcriptase | 1μl |
|  | *Total* | *7μl* |

* + - Aliquot master mix I to strip tubes, mix by pipetting, pulse spin
    - Make sure both master mixes stay very cool, as the next steps to get RNA from main lab will take some time
    - Bring 2º container and small red biohazard bag (under sink) to main lab  
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    - Dispose gloves and change to hall gloves
    - Put biohazard bag on clean cart
    - Fill secondary containment bin with ice from autoclave room (Briggs 252)
    - Take bin and bag to main lab
  + In main lab (Briggs 255)
    - Remove samples from 80ºC freezer
    - Wipe down samples with 10% bleach in chemical hoodA picture containing indoor, table, kitchen, sitting

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    - Let thaw on ice and bring to plate centrifuge
    - Spin @ 2250 rcf, 40”
    - Put samples back on ice and wipe down centrifuge interior (anything that potentially came into contact with sample) w/ 10% bleach
    - Put samples on cart, dispose gloves in red biohazard bag
    - Use new gloves to bring cart to flu room
  + In flu room (Briggs 257)
    - Change gloves and don flu room PPE
    - Wipe down secondary containment bin with 10% bleach before putting in biosafety cabinet
    - Put iced samples into clean biosafety cabinet
    - Add RNA to strip tubes
    - Incubate samples 65ºC, 5’ (program SARSCOV2 > CDNA1 on PCR machine)
    - Ice samples 1min
    - Add master mix II to strip tubes, 7µL per sample, mix by pipetting, pulse spin
    - Incubate samples in PCR machine with the following cycling conditions:

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|  | **Temperature** | **Time** |
|  | 23ºC | 10’ |
|  | 50-55ºC | 10’ |
|  | 80ºC | 10’ |
|  | 5ºC | Hold |

* + - SAFE STOPPING POINT, FREEZE -20ºC if >3 DAYS OR 4ºC if < 3 days
    - Note plate/tube label and location. Default location is the bottom shelf of the -20ºC freezer in main lab (Briggs 255)
  + All subsequent steps in the protocol can be conducted in the main lab, as they do not pose a biosafety risk.
* **Primer pool preparation (only if aliquots are not available):** Working aliquots of both nCov-2019/v3 primer pools (10μM) are in bottom shelf of -20ºC freezer, Box 3.
  + - * In biosafety cabinet thaw nCov-2019/v3 primers (100μM stocks)
      * Make 10μM aliquots of each pool (1 and 2)

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|  | **Component** | **Volume** |
|  | Molecμlar grade water | 180μl |
|  | Pool 1 OR Pool 2 | 20μl |
|  | *Total* | *200μl* |

* **Multiplex PCRs *(1.5 hours + PCR run time)***
  + - This protocol calls for two separate multiplex PCRs per sample, one is done with primer Pool 1, the other with primer Pool 2.
    - Make sure to Carefully label 2 sets of strip tubes with sample ID and pool (different colored markers and separate tube racks for each pool) and set up multiplex PCR in strip tubes
    - Make master mix for each pool in microcentrifuge tubes

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|  | **Component** | **Pool 1** | **Pool 2** |
|  | 5X Q5 Reaction buffer | 5μl | 5μl |
|  | 10mM dNTPs | 0.5μl | 0.5μl |
|  | Q5 Hot Start DNA Polymerase | 0.25μl | 0.25μl |
|  | V3 Primer Pool 1 or 2 (10μM) | 4μl | 4μl |
|  | Nuclease-free water | 12.75μl | 12.75l |
|  | *Total* | *22.5μl* | *22.5μl* |

* + - Aliquot master mix to strip tubes
    - Wipe down tubes and biosafety cabinet with 70% ethanol
    - Wipes down strip tubes and put in biosafety cabinet
    - Pulse spin cDNA
    - Add 2.5μl cDNA to master mix and pipet to mix
    - Pulse spin samples
    - Setup PCR machine

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|  | **Step** | **Temperature** | **Time** | **Cycles** |
|  | Heat Activation | 98ºC | 30” | 1 |
|  | Denaturation | 98ºC | 15” | 25-35 |
|  | Annealing | 65ºC | 5’ | 25-35 |
|  | Hold | 4ºC | indefinite | 1 |
| *(Note: 25 cycles for Ct 18-21, up to 35 cycles for Ct ≥ 35)* | | | | |

* + - SAFE STOPPING POINT, FREEZE -20ºC if >3 DAYS OR 4ºC if < 3 days
* **Qubit samples to check concentration (*this step is optional at this point, can return to check individual samples as needed*)**
  + - * Make working solution *(# of samples + 2 standards)*

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|  | **Component** | **Volume** |
|  | Qubit buffer | 199μl |
|  | Qubit reagent | 1μl |
|  | *Total* | *200μl* |

* + - * Vortex and add 190μl working solution to standard tubes
      * Add 10μl of standard to appropriate tube, vortex 2-3”
      * Add 199μl working solution to new sample tubes
      * Add 1μl sample to appropriate tube, vortex 2-3”
      * Incubate @ RT, 2’
      * Press **DNA** > **dsDNA High Sensitivity** > **Read Standards**
      * Insert Standard #1, close lid, press **Read standard**, remove Standard #1
      * Insert Standard #2, close lid, press **Read standard**, remove Standard #2
      * Press **Run samples**
      * Select sample volume and units

Insert sample tube, close lid, press **Read tube**, record and remove sample *(repeat for all samples)*

* **Pool PCRs and Concentrate Products *(1.5 hours)***
  + - Here we will bring together the separate Pool 1 and Pool 2 PCR products by sample.
    - Label a new strip tube by sample.
    - Using a multichannel, pipette (20µL) of each Pool 1 PCR product into a new strip tube carefully to ensure sample identity is retained.
    - Using a multichannel pipette, transfer (20µL) of each Pool 2 PCR product into the strip tube already containing the Pool 1 aliquot for a total of 40µL

* + - Follow directions for size selection and purification using the Zymo Research Select a Size and DNA Concentrator kit and protocol, with the following notes:
      * Use the ≥ 100 bp cutoff, use 10µL for final elution, incubate column with elution buffer for 5 minutes before final spin
* **Native barcoding (one-pot approach)**
  + - Assemble the following master mix in a new microcentrifuge tubes and aliquot to strip tubes

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|  | **Component** | **Volume** |
|  | Nuclease-free water | 7.5μl |
|  | Ultra II End Prep Reaction Buffer | 1.75μl |
|  | Ultra II End Prep Reaction Enzyme mix | 0.75μl |
|  | *Total* | *10μl* |

* + - Add 5μl of concentrated PCR product
    - Incubate samples

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|  | **Temperature** | **Time** |
|  | RT | 10’ |
|  | 65ºC | 10’ |
|  | ice | 1’ |

* + - In a new microcentrifuge tube assemble the following master mix and aliquot to strip tubes

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|  | **Component** | **Volume** |
|  | Nuclease-free water | 5.7μl |
|  | Ultra II Ligation Master mix | 10μl |
|  | Ligation Enhancer | 0.3μl |
|  | *Total* | *16μl* |

* + - Add 2.5μl NBXX barcode *(use 6-24 barcodes in a library)*
    - Add 1.5μl previous reaction mixture
    - Incubate samples

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|  | **Temperature** | **Time** |
|  | RT | 20’ |
|  | 65ºC | 10’ |
|  | ice | 1’ |

SAFE STOPPING POINT, FREEZE -20ºC if >3 DAYS OR 4ºC if < 3 days

* + - In new LoBind microcentrifuge tube, pool all 20μl one-pot barcoding reactions *(can use same pipette tip or use a sterile reservoir located in the bottom right drawer of the post-PCR bench)*
    - Add 0.4x volume SPRI beads (aka AMPure XP beads, located in 4ºC lab frigde in fridge room) to tube, mix by gently flicking or pipetting
    - Pulse spin
    - Incubate 5’ @ RT
    - Place on magnetic rack (located on first overhead shelf of post-PCR bench) 2’ or until supernatant is completely clear
    - Carefully remove and discard supernatant *(do not touch pellet)*
    - Add 700μl SFB, resuspend by pipetting
    - Pulse spin
    - Place on magnetic rack, let clear. Remove and discard supernatant
    - Add 700μl SFB, resuspend by pipetting
    - Pulse spin
    - Place on magnetic rack, let clear. Remove and discard supernatant
    - Pulse spin to remove residual SFB *(do not air dry)*
    - Add 200μl 70% EtOH
    - Pulse spin
    - Carefully remove and discard EtOH *(do not touch pellet)*
    - Pulse spin and use P10 to remove residual EtOH
    - Leave tube lid open for 1’ or until it loses shine *(do not let it dry completely or it will crack and be hard to resuspend)*
    - Resuspend pellet in 30μl EB, mix by gentle flicking or pipetting
    - Incubate 2’ @ RT
    - Place on magnetic rack and transfer supernatant to a new lo-bind microcentrifuge tube
    - Qubit to check cleanup efficiency
    - Setup AMII adapter ligation master mix in new microcentrifuge tubes

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|  | **Component** | **Volume** |
|  | NEBNext Quick Ligation Reaction Buffer (5X) | 10μl |
|  | Adapter mix (AMII) | 5μl |
|  | Quick T4 DNA Ligase | 5μl |
|  | *Total* | *20μl* |

* + - Add master mix to sample
    - Incubate 20’ @ RT
    - Add 50μl (1:1) SPRI beads to sample, mix by gently flicking or pipetting
    - Pulse spin
    - Incubate 5’ @ RT
    - Place on magnetic rack 2’ or until supernatant is completely clear
    - Carefully remove and discard supernatant *(do not touch pellet)*
    - Add 250μl SFB, resuspend by pipetting
    - Pulse spin
    - Remove and discard supernatant
    - Add 250μl SFB, resuspend by pipetting
    - Pulse spin
    - Remove and discard supernatant
    - Pulse spin to remove residual SFB *(do not air dry)*
    - Add 15μl EB, resuspend by pipetting
    - Incubate 2’ @ RT
    - Place on magnetic rack until clear
    - Transfer supernatant to new lo-bind microcentrifuge tubes *(can store in 10mM Tris pH 8 @ 4ºC for up to a week)*
    - Qubit sample *(see previous)*
* **MinION sequencing**
  + - Prime flowcell insert location and load 20ng library
      * Thaw following reagents insert location @ RT, then put on ice
        + Sequencing buffer (SQB)
        + Loading beads (LB)
        + Flush buffer (FLB)
        + Flush tether (FLT)
      * Add 30μl FLT to FLB, vortex to mix
      * Place new flow cell on MinION *(note pores and flow cell number)*
      * Rotate inlet port cover clockwise 90º so priming port is visible
      * With P1000, volume @ 800μl, place tip in inlet port and turn volume dial increasingly to remove air *(do not remove so much volume so that air is introduced onto the rectangular array via outlet)*
      * Load 800μl FLB *(plus FLT)* into flow cell via inlet port ***slowly*** to avoid bubbles
      * Wait 5’
      * Gently lift SpotON cover to open SpotON port
      * Load another 200μl FLB *(plus FLT)* into flow cell via inlet port to initiate siphon at SpotOn port to load library dilution
      * In new microcentrifuge tubes prepare library dilution *(mix LB immediately before use)*

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|  | **Component** | **Volume** |
|  | SQB | 37.5μl |
|  | LB | 25.5μl |
|  | Final library (20ng total) | 12μl |
|  | *Total* | *75μl* |

* + - * Mix by gently pipetting
      * Add 75μl library dilution to flow cell via SpotON port dropwise *(make sure each drop siphons into port before adding the next)*
      * Gently replace SpotOn port cover, making sure the bung enters the SpotOn port, close inlet port and close MinION lid
    - Start sequencing run using MinKNOW
      * Plug MinION into computer and wait for MinION and flowcell to be detected
      * Choose flow cell 'FLO-MIN106' from the drop-down menu.
      * Then select the flowcell so a tick appears.
      * Click the 'New Experiment' button in the bottom left of the screen
      * On the New experiment popup screen, select the running parameters for your experiment from the individual tabs:
        + Experiment: Name the run in the experiment field, leave the sample field blank.
        + Kit: Selection: Select LSK109 as there is no option for native barcoding (NBD104).
        + Run Options: Set the run length to 6 hours (you can stop the run once sufficient data has been collected as determined using RAMPART).
        + Basecalling: Leave basecalling turned on/off? but select 'fast basecalling'.
        + Output: The number of files that MinKNOW will write to a single folder. By default this is set to 4000 but can be reduced to make RAMPART update more frequently.
      * Click 'Start run'
      * Monitor the progess of the run using the MinKNOW interface