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🌐 Rapid Tiled Amplicon Sequencing of Sars-CoV-2 using Illumina's DNA Prep

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| 1 | Works for me | dx.doi.org/10.17504/protocols.io.bnnbmdan |

Coronavirus Method Development Community

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ABSTRACT

A protocol for rapid, low-throughput (24 samples or fewer) sequencing of Sars-CoV-2-containing samples using Illumina's DNA prep, and the ARTIC protocol for tiled amplicon sequencing. Turnaround time for this protocol from raw sample to sequence data is ~35 hours, making it ideal for use when rapid elucidation of samples is necessary.

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ABSTRACT

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cDNA synthesis

1   For cDNA synthesis, follow steps 1-6 of the v2 of nCoV-2019 sequencing protocol by Josh Quick
    dx.doi.org/10.17504/protocols.io.bdp7i5rn .

A new version of this protocol has been released which replaces ThermoFisher's SSIV reverse transcriptase with NEB's Lunascript. Though this provides significant cost savings, in our limited trial, the SSIV RT significantly

outperformed the Lunascript.

⊠ Superscript IV Reverse Transcriptase **Life Technologies Catalog #18090050**

⊠ dNTP Mix (10 mM each) **Thermo Fisher Catalog #R0191**

⊠ RNaseOUT™ Recombinant Ribonuclease Inhibitor **Invitrogen - Thermo Fisher Catalog #10777019**

⊠ Random Hexamers (50 µM) **Thermo Fisher Catalog #N8080127**

We did not dilute based on CT value prior to conversion, and noted no issues with inhibition.

ARTIC Gene-specific PCR

2 GS amplification with ARTIC primers is based on version 2 of the nCoV-2019 protocol by Josh Quick (dx.doi.org/10.17504/protocols.io.bdp7i5rn) with modifications.

Using cDNA obtained in step 1, follow steps 6-9 of the ARTIC protocol above to complete gene-specific PCR. **35 cycles should be used for PCR**

⊠ Q5 High-Fidelity 2X Master Mix - 500 rxns **New England Biolabs Catalog #M0492L**

3 Following gene-specific PCR, for each sample, combine full volume of reaction 1 with full volume of reaction 2, for a total of 50ul.

4 Perform 1 1X Ampure XP bead cleanup as follows:

⊠ Agencourt AmPure XP beads **Contributed by users Catalog #A63880**

5 Pour beads into reservoir for use with a 96 well plate.

6 For each reaction, pipette 50 uL beads into the reaction wells.

7   Mix by slowly pipetting up and down at least 10 times. Make sure solution is completely homogenous.

8   Let the mixture sit on the bench for 5 minutes for the beads to bind the DNA.

9   Place the plate on a magnetic plate and let it stand for 5 minutes for the beads to collect on the bottom of the plate.

10   Discard the liquid from wells without touching the beads.

11   Dispense 200µl 80% ethanol into each well without disturbing beads while the plate is sitting on a magnetic plate.

12   Incubate for 30 seconds. If you disturbed the beads, then let the mixture stand for 2 minutes.

13   Discard the Ethanol without touching the beads.

14   Dispense another 200µl 80% ethanol into each well, without disturbing beads, while the plate is sitting on a magnetic plate.

15   Incubate for 30 seconds. If you disturbed the beads, then let the mixture stand for 2 minutes.

16   Discard the ethanol without touching the beads. Make sure all ethanol is discarded as it can interfere with DNA elution.

17   Place the plate on the bench and air dry beads for ~5 minutes or until beads appear dry (over-dried beads appear as a cracked, dried surface).

18   Pipette 32.5 uL of pre-warmed 10mM Tris HCL, with 0.05% Tween 20 onto dry wells and pipette beads up and down to bring them into solution.

19   Place beads on a magnetic plate and let beads collect for 3 minutes.

20 Remove 30ul (leaving behind the 2.5uL extra) of eluted DNA into a new PCR reaction plate without touching the beads. The beads have no more DNA bound to them.

QC of PCR product

21 Qubit each sample and determine concentration post-cleanup.

⊠ Qubit dsDNA HS (High sensitivity) Assays **Thermo Fisher Scientific Catalog #Q32851**

⊠ Qubit Fluorometer **Life Technologies Catalog #Q33216**

22 In a new plate or 8-strip tube, dilute each sample as necessary with MBG H20 so that total input amount going into Illumina DNA Prep is 100-500ng, and final sample volume is 30ul.

> For high-CT samples, it may not be possible to input 100ng. If this is the case, aim for the highest input possible without using all of gene-specific product (in the event that troubleshooting is necessary).

Illumina DNA prep- tagmentation

23 Steps 23-58 detail the protocol for Illumina's DNA Prep. The full protocol can be found here:
https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/illumina_prep/illumina-dna-prep-reference-guide-1000000025416-09.pdf

Prior to beginning Illumina DNA prep, bring the following reagents to room temperature. **EPM should be thawed, but remain on ice throughout protocol.**

Reagent	Storage
BLT	2C-8C
TB1	-25C
Index Adapter Plate	-25C
Sample Purification Beads	2C-8C
RSB	-25C

24 Vortex BLT thoroughly, and combine the following reagents to prepare the tagmentation master mix. Reagent overage is included.

Reagent	Volume
BLT	11
TB1	11

25 Vortex the mastermix, and add 20ul master mix to the 30ul diluted DNA from step 22. Pipette to mix. Cap tubes and seal plate, but **do not centrifuge**. Bead mixture needs to remain suspended.

26 Run the following program on a thermal cycler, with heated lid set to 100C.

Temperature	Time
55C	15 minutes
10C	hold

27 Remove plate from thermal cycler. Add 10ul TSB to each reaction. Slowly pipette to resuspend beads. Seal plate. **Do not centrifuge**.

28 Run the following program on a thermal cycler, with heated lid set to 100C.

Temperature	Time
37C	10 minutes
10C	hold

Illumina DNA prep- tagmentation clean-up

29 Remove seal, and place the plate on a magnetic stand. Wait until liquid is clear (~1-3 minutes)

30 Using a multichannel pipette, remove supernatant and discard.

31 Remove plate from magnet. Add 100ul TWB, pipetting very slowly to resuspend beads to avoid foaming.

32   Place the plate back on the magnetic stand, and wait until liquid clears (1-3 minutes).

33   Using a multichannel pipette, remove supernatant and discard.

34   Repeat steps 31-33 for a total of 2 washes.

35   Add 100ul of TWB to beads. Pipette slowly to resuspend beads to avoid foaming. Place plate back on magnetic stand and proceed to step 36.

Illumina DNA prep- Library amplification

36   While plate is on magnet, make the following master mix. Reagent overage is included in volumes listed.

Reagent	Volume
EPM	22
Nuclease-free water	22

37   Vortex briefly, and centrifuge mastermix for 10 secounds on a table-top centrifuge.

38   Remove supernatant from plate while still on the magnetic stand.

> Some foam may remain, and will not affect library quality.

39   Add 40ul mastermix from step 36 to the beads. Pipette to mix until beads are completely resuspended.

40   Pierce the foil of the adapter index plate with a P200 pipette tip. Add 10ul of pre-prepaired i7 and i5 index adapters from the appropriate wells of the 96 plex plate to the bead mix. Be sure to record which indices are used.

    ⊠ Nextera DNA CD indices **Illumina, Inc. Catalog #20018708**

41 Mix thoroughly by pipetting, seal plate, and centrifuge at 280g for 30 seconds.

42 Place on thermal cycler, and run the following program with heated lid set to 100C

Temperature	Time	Cycles
68C	3 minutes	
98C	3 minutes	
98C	45 seconds	Repeat step 4-6 for a total of 5-12 cycles. Cycle number selection guide is in note below.
62C	30 seconds	
68C	2 minutes	
68C	1 minute	
10C	hold	

PCR cycle number is dependent on original DNA input. Select PCR cycle number based on the following:

DNA input (ng)	Cycle number
1 to 9	12
10 to 24	8
25 to 49	6
50 to 500	5

Illumina DNA Prep- Library purification

43 Centrifuge plate at 280g for 1 minute

44 Place the plate on a magnet and wait for the liquid to clear (5 minutes)

45 Transfer 45 ul of supernatant to a new PCR plate

46   Vortex SPB thoroughly to resuspend

47   Add 81ul of SPB to each well of the plate containing 45ul supernatant. Pipette to mix. Incubate on bench top for 5 minutes to allow beads to bind DNA.

48   Place plate on magnet and wait until the liquid clears (5 minutes)

49   Without disrupting the beads, remove the supernatant and discard.

50   With the plate still on the magnet, add 200u of fresh (<1 week old) 80% ethanol. Do not mix.

51   Incubate for 30 seconds

52   Remove and discard ethanol supernatant.

53   Repeat steps 50-51 for a total of 2 washes.

54   Use a p20 pipette to remove all residual ethanol. Air-dry plate on the magnet stand for 5 minutes, or until beads appear dry, but not cracked.

55   Remove plate from magnetic stand, and add 32ul room-temperature RSB to the beads. Pipette to resuspend.

56   Incubate at room temperature for 2 minutes.

57   Place the plate on the magnetic stand and wait for liquid to clear (2 minutes).

58 Transfer 30ul of supernatant to a new 96-well PCR plate. **These are your final libraries.**

Library QC and pooling.

59 If DNA input to Illumina DNA prep was 100ng or greater for **ALL** libraries, pool equal volumes of each library (up to 24 total) in a new 1.5 ml microcentrifuge tube. **This is your final pool.**

If DNA input of ANY sample was less than 100ng, Qubit all samples.

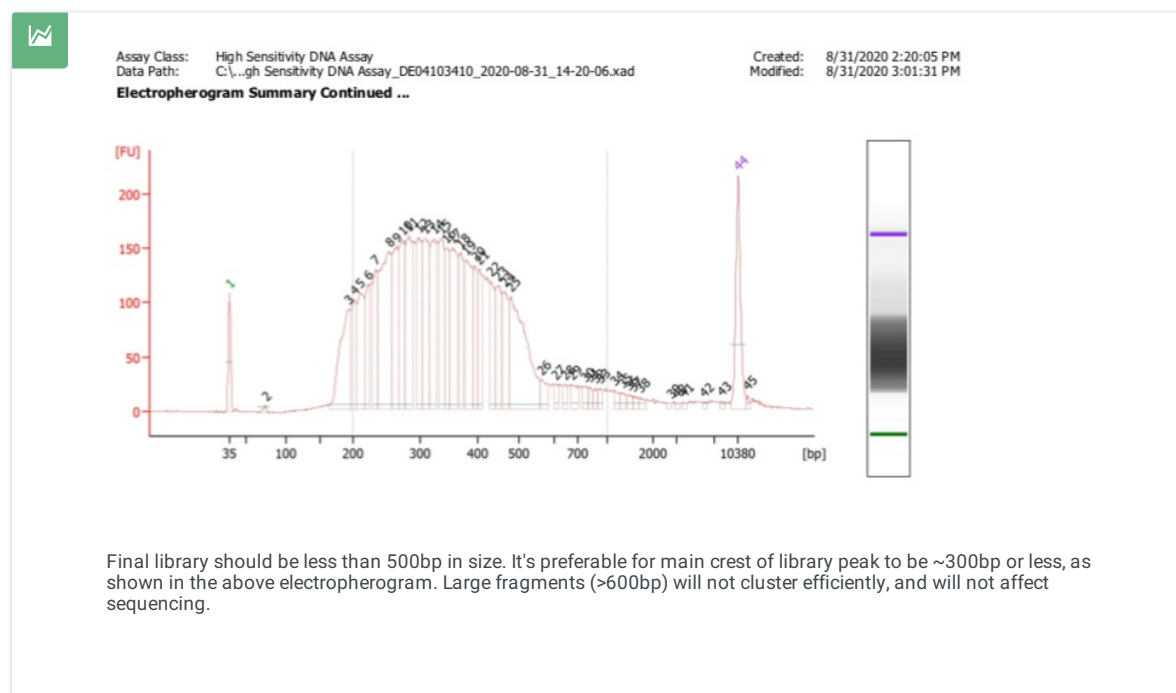⊠ Qubit dsDNA HS (High sensitivity) Assays **Thermo Fisher Scientific Catalog #Q32851**

⊠ Qubit Fluorometer **Life Technologies Catalog #Q33216**

Based on qubit values, combine all samples (up to 24 total) equimolarly in a new 1.5ml microcentrifuge tube. **This is your final pool.**

60 Visualize final pool on a bioanalyzer or tape station to determine final library size.

⊠ Bioanalyzer chips and reagents (DNA High Sensitivity kit) **Agilent Technologies**

Final library should be less than 500bp in size. It's preferable for main crest of library peak to be ~300bp or less, as shown in the above electropherogram. Large fragments (>600bp) will not cluster efficiently, and will not affect sequencing.

You may need to dilute library prior to running high-sensitivity assays. Typically, 1:5 is sufficient to acheive results. Alternatively, use a DNA 1000 kit.

⊠Bioanalyzer chips and reagents (DNA 1000) **Agilent Technologies Catalog #5067-1504**

61 Sequence final pool on a MiSeq v2 nano kit, with 250 X 250 bp paired-end reads, and dual 8bp indexing. Added phiX should be 5% of the total run.

Up to 24 total samples can be sequenced on a single MiSeq nano 500 cycle kit. Sequencing greater than 24 samples will result in insufficient viral genome coverage per sample.

Detailed loading instructions for Illumina MiSeq can be found here:
https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-denature-dilute-libraries-guide-15039740-10.pdf

⊠MiSeq Reagent Nano Kit v2 (500 cycles) **Illumina, Inc. Catalog #MS-103-1003**

⊠phiX V3 control **Illumina, Inc. Catalog #FC-110-3001**

We typically target a cluster density of 600-800k/mm^2. This is slightly less than what Illumina recommends. We've found that having a higher Q30 but less overall data due to lower clustering is more desirable than having a lower Q30 but more overall data, particularly for amplicon sequencing. You can adjust this based on your own preferences.

We found that, for a pool of ~300bp in average size, a loading concentration of 5 pM produced optimal results. Loading concentrations can vary slightly from machine to machine, however, and are dependent on overall library fragment size. You should adjust your loading concentration accordingly.