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High-Throughput Tiled Amplicon Sequencing of Sars-CoV2 using Seqwell's plexWell 384

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bnkimcue

Coronavirus Method Development Community

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cDNA synthesis

1 For cDNA synthesis of viral RNA, follow steps 1-6 of the v2 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**

☒ Random hexamers **Thermo Scientific Catalog #N8080127**

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

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RNaseOUT Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific Catalog #10777019**

We did NOT dilute samples prior to cDNA conversion, and noticed no issues with PCR inhibition by not doing so. Samples were diluted prior to GS pcr. See below.

2 Because the plexWell protocol requires precise sample inputs, cDNA must be diluted prior to gene-specific PCR according to the following:

N2 CT	Dilution
12-15	1:1000
15-18	1:100
18-28	1:10
28+	no dilution

Dilute in 10mM Tris HCl, 0.05% Tween 20, or 10mM Tris HCL.

GS amplification with ARTIC primers

3 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 diluted in step 2, 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**

4 Following GS pcr, combine full volume of reaction 1 with full volume of reaction 2 (final volume, 50ul)

5 Perform one 1X and one 0.7X Ampure XP bead cleanups on the combined PCR product according to the following:

Because plexWell requires precise DNA input amounts, cleanups must be performed to ensure dimer removal for accurate quantitation. Do not simply dilute GS product as in the ARTIC protocol.

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

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

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

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

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

10 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.

11 Discard the liquid from wells without touching the beads.

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

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

14 Discard the ethanol without touching the beads.

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

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

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

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18 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).

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

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

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

22 Repeat steps 5-22, using 35ul beads in step 7 for a second, 0.7X cleanup.

23 Following bead-cleanup, perform a Qubit spot-check of the concentrations of post-GS product across the plate. Typically, quantifying 15-20 samples of a 96-well plate is sufficent to calculate the global dilution factor for the subsequent step.

Qubit Fluorometer **Invitrogen - Thermo Fisher Catalog #Q32866**

Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

Alternatively, if high-throughput quantification methods are available (such as a fluorescent plate reader) quantify the entire plate using a fluorescent assay of choice (such as pico green).

24 ⚠

Average DNA input of samples going into the plexWell protocol should be 2.5ng/ul.

If quantification at step 22 reveals the **average** concentration of samples is greater than 2.5ng/ul, perform the following **plate-wide** global dilution:

Global dilution factor = Average sample concentration/2.5ng

Perform dilution of all samples in plate by the above factor using 10mM Tris HCL. Avoid EDTA-containing solutions when diluting.

The above is taken from Seqwell's protocol note on use of the plexWell 384 kit with Sars-CoV2 sequencing and the

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ARTIC protocol.
https://seqwell.com/wp-content/uploads/2020/05/20200526_plexWell_ARTIC_protocol_20200527.pdf

The DNA inputs for each plate going into the plexWell system must be very precise to ensure correct fragment sizes are obtained at the end of the protocol. Adding too much DNA to the initial tagmentation step can cause fragment sizes to be too large for efficient sequencing on certain short-read Illumina systems.

plexWell384 initial tagmentation and i7 addition

25 Before beginning the plexWell protocol, bring the following reagents to room temperature:

Reagent	Storage conditions	Format
SBP/X96 Plate	-20C	Plate/Red
MAGwise Paramagnetic Beads	4C	Tube
Pool Barcode Reagent	-20C	Tube/Red
Library Primer Mix	-20C	Tube/Natural

plexWell384 **Seqwell Catalog #PW384**

26

Steps 25-67 detail the protocol for Seqwell's plexWell 384 kit. The full user guide can be found here:
https://seqwell.com/wp-content/uploads/2020/02/plexWell_384_and_96_Library_Preparation_Kit_User_Guide.pdf

Protocol deviations from the above pdf will be noted in the steps below.

Add 4ul of gene-specific product (diluted if necessary according to step 23 guidelines) to the appropriate wells of an SBP/X96 plate

Slowly pipette to mix. Try not to introduce excess bubbles.

27 Add 4ul of Coding Buffer into the wells of the SBP/X96 plate

Slowly pipette to mix. Try not to introduce excess bubbles. Seal plate and centrifuge at 1000XG for 1 minute.

At this point, depending on the model of thermal cycler being used, you may need to transfer the contents of the reaction to a mid-skirt plate.

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

Temperature	Time
55C	15 min
10C	hold

29 Centrifuge plate at 1000G for 1 minute to remove condensation from seal.

Dispense 6ul of solution X into each well of the plate.

Slowly pipette to mix. Try not to introduce excess bubbles. Seal plate and centrifuge at 1000XG for 1 minute.

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

Temperature	Time
68C	10min
25C	hold

31 Centrifuge plate at 1000G for 1 minute to remove condensation from seal.

Combine 9ul of each reaction into a 2ml LoBind tube.

plexWell 384 pool purification

32 Vortex MAGwise Paramagnetic Beads thoroughly to ensure beads are resuspended in solution.

33 Add 850 uL (or ~approx. 1 volume equivalent) of MAGwise to the tube with the pooled SB reaction.

34 Pipette mix solution and incubate on benchtop for 5 minutes

35 Place tube on magnetic stand and wait for bead pellet to form (~3 minuntes)

36 Remove and discard supernatant. Do not disturb the pellet.

37 With the beads on the magnet, add two volumes of ethanol. (~1.7mL, or as much as is needed to cover the beads)

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Incubate for 30 seconds

38 Remove and discard supernatant

Complete this step quickly. Do not allow beads to dry out during wash process

39 Repeat steps 37-38 for a total of 2 washes

40 Cap the tube of beads, vortex, and spin briefly to bring residual ethanol to bottom of tube

41 Immediately place on magnet for beads to settle (~30 seconds)

42 Pipette out any residual ethanol

43 Add 40 uL of 10 mM Tris-HCl to bead pellet, remove from magnetic, pipette mix, and incubate on bench top for 5 min

44 Return tube to magnetic stand and wait for bead pellet to form (~2 minutes)

45 Carefully transfer out 39 uL of DNA eluate to a PCR tube [NOTE: This is your PRODUCT]

46 ⚠

Quantify your pool with pico green or Qubit HS reagents.

Pool should quantify between 4-8ng/ul. If you are outside this range, dilute pool to achieve desired concentration. **Correct concentration is essential to obtain products of appropriate size for sequencing.**

Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

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plexWell384 Pool barcoding/i5 addition

47 ⚠

Add **9ul PB** reagent to PCR tube with the purified SB reaction pool. Take note of which pool barcode was used. Pipette thoroughly to mix.

The addition of 9ul PB reagent differs from the Seqwell protocol for the plexWell kit, and is necessary to achieve fragment sizes compatible with short-read sequencing.

This protocol is designed for the high-throughput sequencing of Sars-CoV-2 samples. For multiplexing purposes, ensure that a different pool barcode is used for each plate of 96 (up to 12 uniquely barcoded plates/pools total). Seqwell currently provides 1152 unique index combinations for this purpose.

48 Add 22ul Coding Buffer to the tube and pipette thoroughly to mix. Cap the tube, vortex, and centrifuge briefly.

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

Temperature	Time
55C	15min
25C	hold

50 Briefly centrifuge tube. Add 33ul of X solution to the reaction. Pipette slowly to mix.

Re-cap tube and centrifuge briefly

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

Temperature	Time
68C	10 min
25C	hold

plexWell 384 PB pool purification

52 Vortex and spin PB reaction, and transfer contents to a 1.5ml LoBind tube
Vortex Magwsie beads thoroughly to ensure beads are resuspended in solution

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53 Add 99ul MAGwise beads to the tube with the pooled SB reaction

54 Follow steps 33-41 to clean the SB reaction

55 Add 24ul of 10mM Tris-HCL to bead pellet, remove from magnetic stand, pipette to mix, and incubate on benchtop for 5 minutes

56 Return tube to magnetic stand and wait for bead pellet to form (~2 minutes)

57 Carefully transfer 23ul of DNA eluate to a new PCR tube.

The transfer of some beads with the purified product with not inhibit library amplification

plexWell 384 Library amplification

58 Add 4ul of Library Primer Mix to the purified product

59 Add 27ul of Kapa HiFi Hotstart ReadyMix and mix well by pipetting.
Close PCR tube and centrifuge briefly.

2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #KK2602**

60 Run the following program on a thermal cycler with heated lid set to 100C

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Temperature	Time	Notes
72C	10 min	
95C	3 min	
98C	30 sec	Repeat steps 4-6 for 12 cycles total
64C	15 sec	
72C	30 sec	
72C	3 min	
4C	hold	

plexWell384 Library purification

61 Briefly centrifuge library amplification reaction and measure total volume using a pipette. Transfer product to LoBind tube.

62 Dilute the library to a final volume of 205ul with 10mM Tris-HCL pH 8.0 and pipette to mix

63 Transfer 100ul of diluted library to a new 1.5ml LoBind tube for clean-up. Store the remaining 105ul at -20C until QC is complete.

64 ⚠

Vortex MAGwise beads thoroughly.
Add 75 ul (or ~.75 volumes) of MAGwise beads to the 100 ul of diluted amplified library

This volume differs from the Seqwell protocol for the plexWell kit, and is necessary to achieve fragment sizes compatible with short-read sequencing

65 Follow steps 33-41 to purify library amplification product

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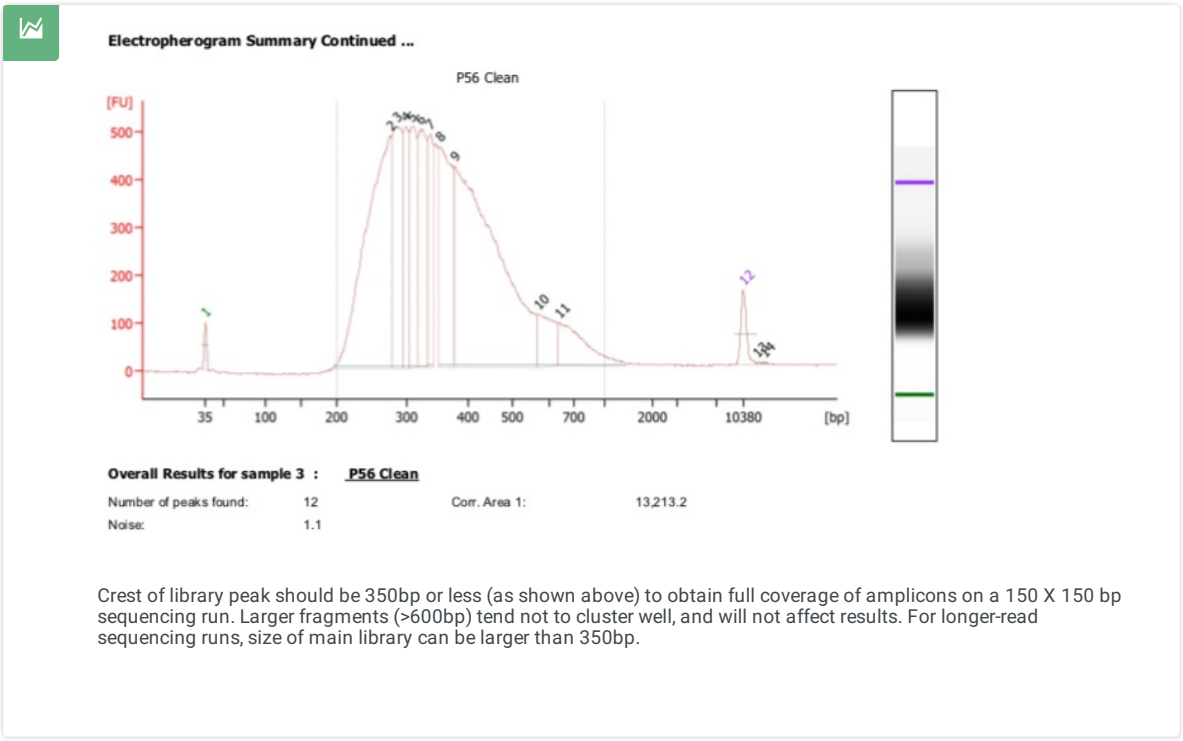
66 Add 32ul 10mM Tris-HCL to bead pellet, remove from magnet, pipette to mix, and incubate on bench top for 5 minutes

67 Return to magnetic stand and wait for bead pellet to form (~2 minutes)

68 Carefully transfer 28ul of DNA eluate to a new 1.5ml LoBind tube. T**his is the final purified library.**

Library QC

69 Visualize final library on a tapestation or bioanalyzer using high-sensitivity reagents.



Crest of library peak should be 350bp or less (as shown above) to obtain full coverage of amplicons on a 150 X 150 bp sequencing run. Larger fragments (>600bp) tend not to cluster well, and will not affect results. For longer-read sequencing runs, size of main library can be larger than 350bp.

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

70 Quantify final library using Kapa Quant reagents and an appropriate real-time PCR machine. Quantify multiple dilutions of each final library to ensure accurate readings.

Alternatively, qubit final library in triplicate to determine concentration.

Qubit dsDNA HS Assay kit **Thermo Fisher Scientific Catalog #Q32854**

KAPA Library Quantification Kit for Illumina® Platforms **Kapa Biosystems Catalog #KK4835**

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Sequencing

71 Pool 12 (up to 1152 samples total) uniquely barcoded pools from steps 1-69 above at equimolar concentrations

72 Visualize final pool of pools on a bioanalyzer or fragment analyzer to determine final pool size. Pool may need to be diluted prior to running on HS kit. Alternatively, if expected concentration is high enough, DNA 1000 reagents may be used.

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

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

73 Quantify final pool of pools using Kapa Quant reagents and an appropriate real-time PCR machine. Quantify multiple dilutions of each final library to ensure accurate readings.

Alternatively, qubit final library in triplicate to determine concentration.

KAPA Library Quantification Kit for Illumina® Platforms **Kapa Biosystems Catalog #KK4835**

Qubit dsDNA HS Assay kit **Thermo Fisher Scientific Catalog #Q32854**

74 Sequence pool of pools (1152 samples total) on a NextSeq High-Output kit, using 150 X 150 bp paired end reads, and 8bp dual index reads. Added PhiX should be 5% of the total run.

Detailed loading instructions for Illumina NextSeq500/550 can be found here: https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/nextseq/nextseq-denature-dilute-libraries-guide-15048776-09.pdf

We typically target a cluster density of 160-200k/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 ~350bp in size, a loading concentration of 1.5 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.

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NextSeq High Output Kit **Illumina, Inc. Catalog #20024908**

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

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