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MiniXT protocol

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ABSTRACT

We present the 'mini-XT' miniaturized tagmentation-based library preparation protocol used for Illumina WGS of SARS-CoV-2 positive samples. Reverse transcription and amplification is based upon the nCoV-2019 sequencing protocol v3 (LoCost)V.3 by Josh Quick.

The key new feature of the protocol is the use of acoustic liquid transfer to automate and reduce volumes during library preparation. It is optimized for the sequencing of 384 samples, offering reduced consumable use and costs and improved throughput.

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Whole genome sequencing, WGS, Illumina, Nextera, SARS-CoV-2, COVID19, NEB, Echo, acoustic liquid transfer

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DISCLAIMER:

cDNA synthesis 1h 30m

1 Thaw the RNA samples on ice.

2 Shake the plates at 1200 RPM for 10 seconds, and spin at 280 xg for 1 min.

3 Thaw LunaScript RT Supermix and nuclease free water on ice and at RT, respectively.

4 Prepare the mastermix as show in Table 1 below:

5

A	B	C
Reagent	**For 1 reaction (µL)**	**For 420 reactions (µL, 4 x 96 well plate)**
Luna RT Supermix	2	840
Nuclease free water	3	1260

Table 1: RT mastermix.

6 Add 5 µL mastermix to each well of a 95-well plate, followed by 5 µL RNA. Each RT plate must contain two no template controls (NTCs) in the form of 5 µL of nuclease free water. Pipette mix ten times upon addition.

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7 Spin the RT plate(s) at 1000 xg for 1 min.

8 Incubate on a thermocycler using the conditions outlined in Table 2, ensuring that the lid is heated to 105 ºC: 23m

00:23:00

A	B	C
Step	**Temperature (°C)**	**Duration**
1	25	2 min
2	55	20 min
3	95	1 min
4	4	∞

Table 2: Cycling conditions for RT.

cDNA amplification 4h 30m

9 Prepare the Q5 PCR mastermixes (A and B, using Artic nCov2019 V3 primers) as outlined in Table 3 and keep on ice.

10

A	B	C
Reagent	**For 1 reaction (µL)**	**For 840 reactions (µL)**
Nuclease free water	12.75	10710
Q5 reaction buffer	5	4200
dNTPs	0.5	420
Primers (10 mM: A, or B)	4	3360
Q5 DNA polymerase	0.25	210

Table 3: Q5 PCR mastermix preparation.

11 For one RT plate, prepare one Pool A and one Pool B 96-well plate, where each well contains 22.5 µL of either A or B mastermix.

12 Add 2.5 µL of the RT product from Step 8, and pipette mix ten times.

13 Incubate on a thermocycler using the conditions outlined in Table 4, ensuring that the lid is heated to 105 ºC:

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3h 45m

14 03:45:00

A	B	C
Step	**Temperature (°C)**	**Duration**
1	98	30 sec
2	98	15 sec
3	63	5 min
Repeat steps 1-3 34 times		
4	4	∞

Table 4: Cycling conditions for PCR.

o

Amplicon clean-up 40m

15 Spin Pool A, Pool B 96-well plates at 280 xg, 1 min, RT.

16 In a clean 96-well plate combine 10 µL of Pool A and 10 µL of Pool B. Pipette mix ten times.

17 Spin the A/B plate at 280 xg, 1 min, RT.

18 Add 30 µL of KAPA Pure beads and pipette mix twenty times.

19 Incubate for 5 min, RT.

20 Place the plate on a 96-well plate magnet for 3 min, RT.

21 Remove and discard the supernatant, ensuring that you do not disturb the beads.

22 Keeping the plate on the magnet: perform two ethanol washes as follows:

22.1 Add 140 µL of 80% ethanol to each well.

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22.2 Incubate for 30 sec, RT.

22.3 Remove and discard the supernatant.

23 After the second ethanol wash, air dry the beads for 4 min, RT.

23.1 After 1 min, remove any residual ethanol from the wells.

24 Remove the plate form the magnet, and add 60 µL of 10 mM Tris pH 8.0 to each well. Pipette mix twenty times.

25 Incubate for 5 min, RT.

26 Place the plate on a 96-well plate magnet for 3 min, RT.

27 Transfer the supernatant to a clean 96-well plate, and store at 4 ºC.

Amplicon quantification 30m

28 Spin the plate from Step 27 at 280 xg, 1 min, RT.

29 Prepare the working solution from the Quant-iT™ dsDNA Assay Kit, broad range; for every 200 µL buffer add 1 µL dye. Vortex well to mix.

30 For each plate from Step 27, prepare one black flat-bottom 96-well plate by adding 100 µL of working solution to each well.

31 Prepare one plate for the standards (of which there are eight) by adding 100 µL of working solution in triplicate wells.

32 Vortex and spin down the standards, and add 3 µL to their respective wells.

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33 Add 3 µL of clean amplicons to their respective wells.

34 Measure on a PHERAstar Omega plate reader: shake the plate for 30 sec before reading.

34.1 Read the standards first, generate the second polynomial fit, and save as a template to measure the sample plate(s).

35 Quantify all NTCs using Qubit™ 1X dsDNA HS Assay Kit:

35.1 Prepare the standards:
190 µL of mastermix + 10 µL Standard 1
190 µL of mastermix + 10 µL Standard 2
198 µL of mastermix + 2 µL Control.

35.2 For each NTC:
198 µL of mastermix + 2 µL NTC.

35.3 Vortex and incubate for 2 min, RT, in the dark.

35.4 Read on a Qubit 4 Fluorometer.

36 Quantify all NTCs using TapeStation 4200 and Aligent D1000 screentape and reagents:

36.1 To each 0.2 mL TapeStation 4200 eppy, add 3 µL sample buffer.

36.2 Add 1 µL ladder or 1 µL NTC to the appropriate eppys.

36.3 Vortex and spin down.

36.4 Measure on TapeStation 4200 as per manufacturer's guidelines.

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Amplicon normalisation 1h 15m

37 Prepare a .csv file containing:
Echo plate position,
Plate ID (A1, A2, B1, or B2),
Well position on plate,
Sample IDs,
Quant-IT readings,
and volume (in nL) to add to obtain amplicons at 0.2 ng/µL.

38 The maximum volume Echo can transfer is 10000 nL, therefore any samples with low Quant IT readings yielding volumes > 10000 nL must be changed to 10000 nL.

39 Change NTC transfer volumes to 500 nL.

40 Combine 30 µL from each well of the four 96-well plates into an Echo qualified flat-bottom 384-well polypropylene microplate 2.0 in the A1, A2, B1, B2 pooling style as shown in Table 5:

41

A	B	C	D
96-well plate (row)	**A1 (384 position)**	**96-well plate (row)**	**A2 (384 position)**
A	A1 (A3, A5...)	**A**	A2
B	C1 (C3, C5...)	**B**	C2
C	E1 (E3, E5...)	**C**	E2
D	G1	**D**	G2
E	I1	**E**	I2
F	K1	**F**	K2
G	M1	**G**	M2
H	O1	**H**	O2
96-well plate (row)	**B1 (384 position)**	**96-well plate (row)**	**B2 (384 position)**
A	B1	**A**	B2
B	D1	**B**	D2
C	F1	**C**	F2
D	H1	**D**	H2
E	J1	**E**	J2
F	L1	**F**	L2
G	N1	**G**	N2
H	P1	**H**	P2

Table 5: Combining four 96-well plates into one 384-well plate.

42 Perform a plate survey on the 384-well plate from Step 40, and perform focus calibration on the Echo.

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43 Label a new Echo qualified flat-bottom 384-well polypropylene microplate 2.0 as "0.2 ng/µL" and use the .csv file from Step 37 as your Echo "region definitions" to perform amplicon transfer.

44 For transfer exceptions, either: (a) create a rescue file, or (b) manually transfer to 0.2 ng/µL Echo qualified flat-bottom 384-well polypropylene microplate 2.0.

45 To each well of 0.2 ng/µL Echo qualified flat-bottom 384-well polypropylene microplate 2.0, add 40 µL10 mM Tris pH 8.0 and pipette mix eight to ten times.

46 Spin the plate from Step 45 at 280 xg, 1 min, RT, and store at 4 ºC.

Library preparation for Illumina sequencing 2h 30m

47 Thaw Nextera XT DNA Library Preparation Kit reagents and decant into the following Echo source plates:

47.1 2 mL TD buffer into one well of an Echo 525 liquid handler source plate.

47.2 63 µL of ATM into A1, B1, to H1 (inclusive) of an Echo qualified flat-bottom 384-well polypropylene microplate 2.0.

47.3 Full volume of NT buffer into one well of an Echo 525 liquid handler source plate.

47.4 Full volume of NPM buffer into one well of an Echo 525 liquid handler source plate.

48 Perform focus calibration and plate surveys on all Echo qualified flat-bottom 384-well polypropylene microplate 2.0 (as in Step 42).

49 Using the Echo, perform the following sequential transfers:

49.1 1 µL of TD buffer to each well of an Eppendorf Twin tec PCR plate, 384 wells.

49.2 500 nL of amplicons (From Step 46) to corresponding wells of an Eppendorf Twin tec PCR plate, 384 wells.

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49.3 500 nL of ATM to each well of an Eppendorf Twin tec PCR plate, 384 wells.

49.4 For transfer exceptions, see Step 44.

49.5 Incubate on a thermocycler for 5 min, at 55 ºC.

49.6 Transfer 500 nL of NT buffer to each well of an Eppendorf Twin tec PCR plate, 384 wells.

49.7 Incubate for 5 min, RT.

49.8 Transfer 1 µL of pre-mixed forward and reverse indexes (or 500 nL of each seperately) to corresponding wells of an Eppendorf Twin tec PCR plate, 384 wells.

49.9 Transfer 1.5 µL of NPM to each well of an Eppendorf Twin tec PCR plate, 384 wells.

49.10 Incubate on a thermocycler using the conditions outlined in Table 6, ensuring that the lid is heated to 105 ºC:

49.11

A	B	C
Step	**Temperature (°C)**	**Duration**
1	72	3 min
2	95	30 sec
3	95	10 sec
4	55	30 sec
5	72	30 sec
6	72	5 min
7	4	∞

Table 6: Cycling conditions for library preparation.

50 Spin the plate from Step 49 at 280 xg, 1 min, RT, and store at 4 ºC.

Library QC 2h 30m

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51 Label four 1.5 mL low-binding eppys:

"Pool ID"
“First clean”
“Second clean”
"Pool ID CLEAN”

52 Pool 2.5 µL from each well of the plate from Step 50 into one row of a 96-well plate.

53 Pool each well from this row into the "Pool ID" eppy, and pipette mix ten times.

54 Transfer 50 µL to "First clean" eppy.

55 Add 80 µL KAPA Pure beads and pipette mix twenty to thirty times.

56 Incubate for 10 min, RT.

57 Place the eppy on a magnetic stand for 2-5 min until the supernatant is clear.

58 Remove and discard the supernatant.

59 Keeping the eppy on the magnetic stand, wash beads twice with 80% EtOH. For each wash:

Add 400 µL to the eppy,
Incubate for 30 sec to 1 min, RT,
Remove and discard the supernatant

60 Air dry beads for 8 min, RT.

61 Add 50 µL 10 mM Tris pH 8.0 and pipette mix ten times.

62 Incubate for 10 min, RT.

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63 Place the eppy on a magnetic stand for 2-5 min until the supernatant is clear.

64 Transfer elute to "Second clean" eppy.

65 Repeat Steps 55 to 63.

66 Transfer elute to "Pool ID, CLEAN" eppy.

67 Quantify the cleaned pool (in duplicate) using Qubit™ 1X dsDNA HS Assay Kit as in Step 35.

68 Quantify the cleaned pool using TapeStation 4200 and Aligent D1000 screentape and reagents as in Step 36.

Illumina sequencing 1d 2h

69 Sequencing is performed with a MiSeq Reagent Kit v2) (300 cycles PE):

69.1 Thaw the contents of Box 1 of 2 1h before use.

69.2 Store Box 2 of 2 at 4 ºC until use.

70 Prepare the MiSeq sample sheet as follows:

70.1 Add Pool ID to "Experiment Name", and "Sample Project" column.

70.2 Add Sample IDs from Step 37. to "Description" column.

70.3 Add sample barcodes to "Sample ID" column.

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70.4 Add the plate ID to "Sample Plate" column.

71 Calculate the molarity of the pool, and the amount of pool and 10 mM Tris pH 8.5 (in µL) required to dilute to 4 nM, using:
(a) the Qubit measurements for the Pool (from Step 67),
(b) the average fragment size (bp) from the TapeStation (From Step 68),
and the following formula: =(1000000*(1/average fragment size)*(1/660))*AVERAGE(Qubit measurments)

72 Prepare the pool for the MiSeq as outlined below:

72.1 Prepare the 4 nM pool using the values obtained in Step 71. Vortex and spin down.

72.2 Prepare 0.2 N NaOH.

72.3 In an eppy labelled "20 pM" combine 5 µL of 4 nM pool and 5 µL0.2 N NaOH.

72.4 Incubate for 5 min, RT.

72.5 Add 990 µL HT1 buffer to 20 pM eppy. Vortex and spin down.

72.6 In an eppy labelled "9 pM" combine 330 µL HT1 buffer and 270 µL from the 20 pM eppy. Vortex and spin down.

72.7 In an eppy labelled "Load" combine 594 µL from the 9 pM eppy with 6 µLf 12.5 pM PhiX (control template). Vortex and spin down.

72.8 Add 600 µL from Step 72.7 to the reagent cartridge as per manufacturer's guidelines.

73 Prepare the flow cell, incorporation buffer as per manufacturer's guidelines, and prepare the MiSeq as instructed on the screen.

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74 Perform Basecalling and Demultiplexing of the sequencing data using the Illumina "MiSeq Reporter software" (or alternatively the Illumina "bcl2fastq" or "BCL Convert").

75 Use the "connor-lab/ncov2019-artic-nf" nextflow-based open-source pipeline, as used by the COG-UK consortium, (from: https://github.com/connor-lab/ncov2019-artic-nf) to perform the QC, read-trimming, alignment to Wuhan reference, and generate consensus fasta sequence. Use the '--profile conda' option with the '--illumina' option

76 Use the Pangolin open-source software (from: https://github.com/cov-lineages/pangolin) to call the Lineage for each sample's consensus fasta sequence.

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