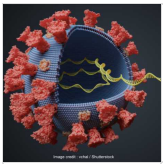

May 07, 2021

ARTIC NEB Tagmentation protocol - high throughput whole genome sequencing of SARS-CoV-2.

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1 *Works for me* This protocol is published without a DOI.

Coronavirus Method Development Community | Chan Zuckerberg Biohub

K Karan Bhatt

ABSTRACT

This protocol is used for sequencing of SARS-CoV-2 in 384 well plate format with miniaturized reaction volumes achieved with automated sample processing using liquid handlers. It has been used for all the SARS-CoV-2 genome sequencing associated with the Chan Zuckerberg Biohub COVID Tracker genomic epidemiology collaboration with California State and County Departments of Public Health from the period of May 2020 to June 2021. Extracted RNA is amplified with ARTIC V3 primers to produce 400 bp amplicons that tile across the SARS-CoV-2 genome. Amplicons are tagmented using homebrew Tn5 transposase loaded with Nextera adapter oligos. The products of tagmentation are amplified with Nextera index primers to generate Illumina libraries for 2 x 150 bp (paired-end) sequencing on the Illumina sequencing platform.

PROTOCOL CITATION

Karan D Bhatt, Amy Kistler, Angela Detweiler, Ashley Byrne, Gabrielle R Lee, G Renuka Kumar, Lienna Y Chan, Lusajo Mwakibete, Michael B, Michelle Tan, Norma Neff, Sabrina A Mann, Sharon Fong 2021. ARTIC NEB Tagmentation protocol - high throughput whole genome sequencing of SARS-CoV-2.. **protocols.io** https://protocols.io/view/artic-neb-tagmentation-protocol-high-throughput-wh-bt66nrhe

KEYWORDS

ARTIC, ARTIC Tagmentation , SARS-CoV-2, SARS-CoV-2 Sequencing

CREATED

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LAST MODIFIED

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PROTOCOL INTEGER ID

49086

MATERIALS TEXT

- **List of liquid handlers used in the process of Library preparation:**
- Hamilton Microlab STARlet Liquid Handling System.
- Agilent Bravo liquid handler.
- Labcyte Echo 550.
- sptlabtech Dragonfly.

- Integra - Integra Biosciences.
- sptlabtech - Mosquito LV.

♦ **Consumables:**

- Biorad 96 well Hard-shell PCR plates - #HSP9601.
- Biorad 384 well clear Hard-shell PCR plates #HSP3901.
- Echo Qualified 384-Well Polypropylene 2.0 Plus Microplate. (# PPL-0200).

♦ **Reagents**

Teknova PCR-Certified Water

- **Teknova Catalog #W3330**

Random Primers **New England**

- **Biolabs Catalog #E7422**

Deoxynucleotide Solution Mix - 40 umol of each **New England**

- **Biolabs Catalog #N0447L**

5X SuperScript Buffer **Invitrogen - Thermo**

- **Fisher Catalog #18-090-200**

10 mM

- DTT **Invitrogen Catalog #18090200**

SuperScript™ IV Reverse Transcriptase **Thermo**

- **Fisher Catalog #18090200**

RNaseOUT RNase Inhibitor **Invitrogen - Thermo**

- **Fisher Catalog #10-777-019**

NEBNext Ultra II Q5 Master Mix - 250 rxns **New England**

- **Biolabs Catalog #M0544L**

1M MgCl2 solution **Thermo Fisher**

- **Scientific Catalog #AM9530G**

NN-Dimethylformamide **Sigma**

- **Aldrich Catalog #227056-1L**

Sodium Dodecyl Sulfate (SDS) **Fisher**

- **Scientific Catalog #BP166-500**

SPRIselect 60 mL **Beckman**

- **Coulter Catalog #B23318**

Input RNA prep - Dilution and plating.

1

- High titer SARS-CoV-2 samples can exhaust free ARTIC primers and form aberrant multimers during multiplex PCR (refer fig. 1). These larger amplicons can cause issues during downstream sequencing that impact genome sequence recovery. Hence, RNA samples need to be diluted by their Cycle Threshold or “Ct” values.

(Note: RNA is temperature sensitive and prone to degradation. Follow RNAse-free practices and avoid multiple freeze-thaw cycles. Always place RNA plates on ice for intermediate steps and processes).

- We usually perform the following steps in a pre-PCR room. This space is free of DNA of any kind and does not allow amplification of DNA. Follow the steps until the multiplex PCR mix and move the plate outside the pre-PCR room for cDNA amplification.

- Use liquid handlers like the Hamilton MICROLAB STARlet or similar to aliqurepresentsot RNA samples from tubes or plates to a barcode-labeled 96 well plate (Biorad hard shell #HSP9601 or similar). Include a minimum of 1 negative control water aliquot on each plate.

- After generating a source plate of original RNA samples, you next need to transfer an aliquot of each sample to a fresh plate for sequencing library prep. Use a Hamilton MICROLAB STARlet or similar liquid handler with a cherry-picking capability to transfer an aliquot of each RNA sample from the source plate into a fresh 'working' 96 well plate. As needed, dilute each individual RNA sample with DNAse/RNAse free water using the following schema:

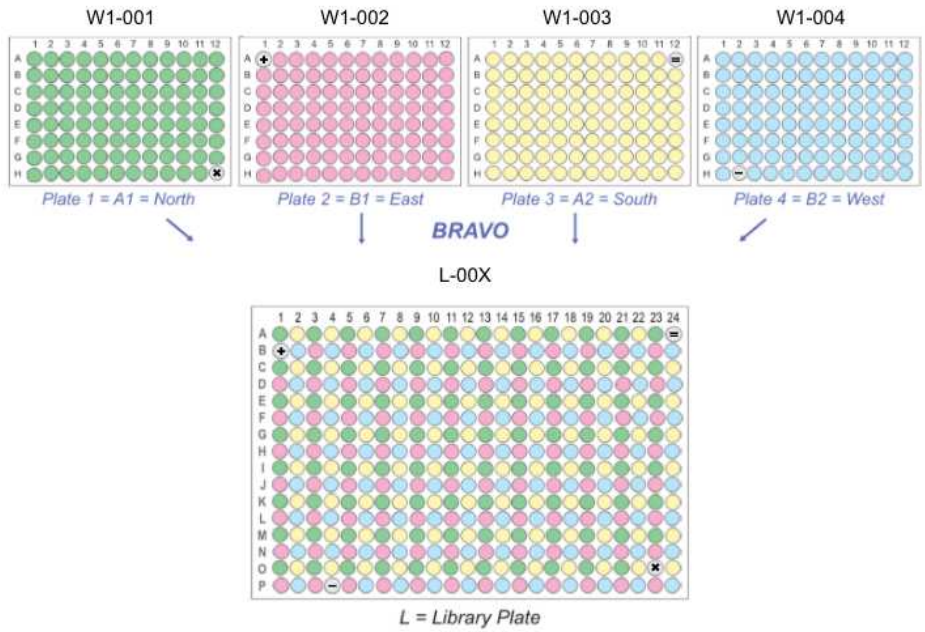
A	B
Cycle threshold values	Dilution
1 - 15	1:10
16 - 35	No dilution

We performed a series of experiments to identify the Ct threshold of 15 for 10X dilution to streamline this sample dilution process without affecting genome recovery. (refer fig. 2)

- Label the final 96 well working plate with a minimum working volume of **14 µl** of each diluted or undiluted sample as “W - barcode”.

- Use the Agilent Bravo or similar liquid handlers to transfer **3 µl** of RNA from 4 x 96 well plates to a 384 well plate (Biorad - #HSP3901) as shown below. Affix a Library plate "L - 00x" barcode to this plate. In the events when you don’t have 384 samples, you can leave the wells empty or fill them with water as a control to trace contamination.

(Note: for the Agilent Bravo, **3 µl** of RNA is transferred to ensure a net of **2.75 µl** is aliquoted to each well.)

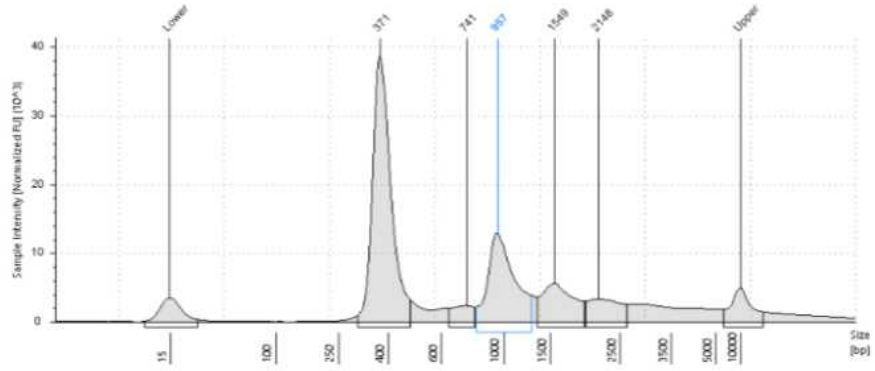


schema to merge 4 x 96 well plates to a 384 well plate. grey wells represent water controls.

♦ Checkpoint: you can potentially store this Library prep plate at -80C. For optimal results continue until cDNA is made.
♦

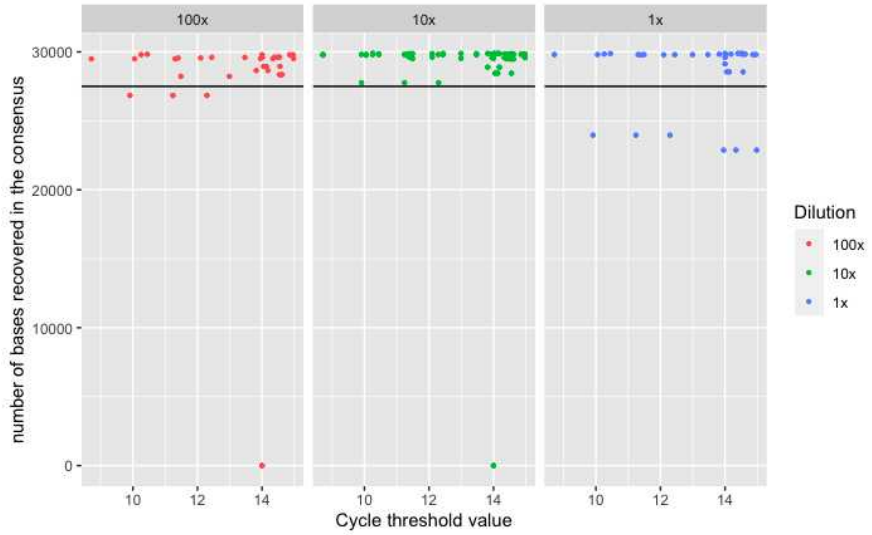
♦ Figures:

- Figure 1: Multimer PCR product formation in multiplex PCR of undiluted high titer samples.



Agilent TapeStation electrophoresis profile of high titer sample. Multimer products from self-annealing and amplification, along with PCR smearing is a result of over-amplification and primer exhaustion.

- Figure 2: genome recovery of RNA samples below Ct value 15 diluted to different extents.



RNA samples below Ct value 15 were diluted 1:10 (10x), 1:100 (100x), or not diluted (1x), then prepped with this protocol for SARS-CoV-2 sequencing. The highest yield of SARS-CoV-2 genome recovery is seen in the 1:10 or 10 x dilution condition.
Note: the black line (y-intercept) on the graph is set to 27500 bases. This corresponds to 92% SARS-CoV-2 genome length recovery.

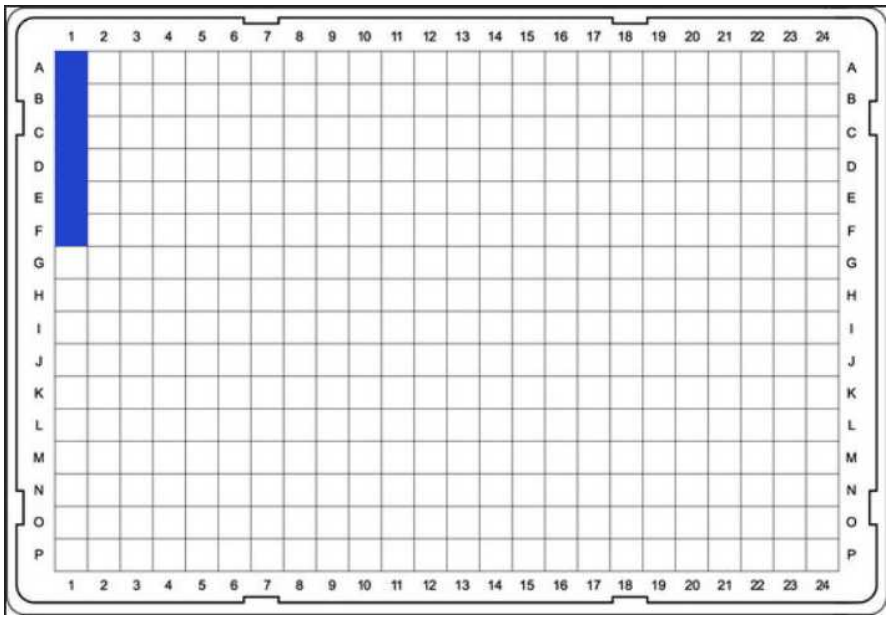
cDNA prep - First strand synthesis.

2 1. dNTPs Hexamer Priming :

- Before starting this step make sure the thermocycler is running the dNTP Hexamer priming program.
- Thaw reagents mentioned in the table on ice. Mix and spin down. Do not vortex enzymes.
- Make the master mix as described in the table inside a PCR hood. alternatively, you can pre-make the master mix and aliquot in single-use Eppendorf tubes (store the pre-made master mix at -20ºC).

A	B	C	D
Reagent	1x reaction volume	400x reaction volume	Notes
1.9 mM NEB Random Primers (E7422AA vial)	0.25	170	70uL added to account for dead volume
10mM dNTP (N0447L)	0.25	170	
RNA Sample from step 1	2.75	-	
Total	3.25	340	

- Add **54.6 µl** of master mix into wells A1-F1 of an Echo Qualified 384-Well Polypropylene 2.0 Plus Microplate. (# PPL-0200)



- Seal the plate, spin down and let the reagents come to room temperature before starting the Echo transfer.
- Use the example protocol attached below as a reference to upload on the Labcyte Echo 550 machine. Using Labcyte Echo 550 or similar, transfer **500 nL** from the source plate to each well in the 384 well Library plate containing **2.75 µl** of RNA.

A	B	C
# source wells:	6	wells
uL/well:	54.6	ul
source well transfer (nL)	500	nL

- Example protocol : **dNTP Hexamer transfer.csv**
- Carefully seal and centrifuge the plate briefly, then place the plate on the thermocycler.

(Note: while the plate incubates in the thermocycler for 5 minutes make your master mix for the reverse transcriptase reaction)

- dNTP Hexamer priming program:

A	B
Program	dNTP Hexamer priming
Lid	105°C
Volume	3.25µL

A	B	C
	Temp	Time
Step 1	65°C	5min
Step 2	4°C	hold

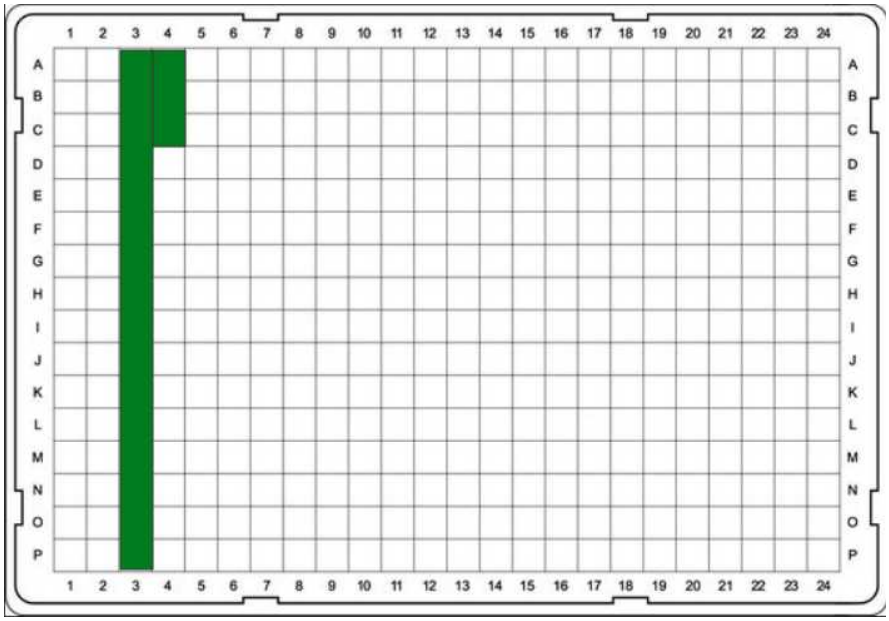
- Immediately cool the plate on ice for a minute and spin down.

3 1. Reverse Transcription Reaction:

- Before starting this step make sure the thermocycler is running the reverse transcription program.
- On ice, thaw reverse transcription reagents in the table below. Mix and spin down. Do not vortex enzymes.
- Make up the master mix as described in the table inside a PCR hood.

A	B	C	D
Reagent	1x Reaction Volume (uL)	400x Reaction Volume	Notes
5x SuperScript IV Buffer (Invitrogen - 18-090-200)	1	645	Master mix is made for 645 reactions to account for dead volume.
100mM DTT (Invitrogen - 18-090-200)	0.25	161.25	
RNaseOUT RNase Inhibitor (Invitrogen - 10-777-019) (40 Units/ul)	0.25	161.25	
SuperScript IV Reverse Transcriptase (Invitrogen - #18-090-200) (200 Units/ul)	0.25	161.25	
Annealed Template RNA from step 2	3.25	-	
Total	5	1128.75	

- Add **55 µl** of master mix into each well for wells A3-P3 and A4-C4 of the same Echo Qualified 384-Well Polypropylene 2.0 Plus Microplate. (# PPL-0200) used in step 2.



- Seal the plate, spin down and let the reagents come to room temperature before starting the Echo transfer.
- Use the example protocol attached below as a reference to upload on the Labcyte Echo 550 machine. Using Labcyte Echo 550 or similar, transfer **1750 nL** from the source plate to each well in a 384 well plate containing **3.25 µl** of Annealed Template RNA.

A	B	C
# source wells:	19	wells
uL/well:	55	ul
source well transfer	1750	nL

- Example protocol : **reverse transcriptase reaction.csv**

- Carefully seal the plate, centrifuge briefly, and place the plate on the thermocycler.
- Reverse transcription program:

A	B
Program	Reverse Transcription
Lid	105°C
Volume	5µL

A	B	C
	Temp	Time
Step 1	25°C	5min
Step 2	42°C	50min
Step 3	70°C	10min
Step 4	4°C	hold

♦ Checkpoint: you can potentially store the cDNA plate at -20C. ♦

Multiplex - cDNA amplification.

4

- The ARTIC primer list (V3) can be found here - https://github.com/artic-network/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V3/nCoV-2019.tsv. These can be purchased in a pool or as individual primers. If purchased as individual primers, resuspend as shown in the notes below:
- ARTIC_Primer_Pooling.docx
- Before starting this step, make sure the thermocycler is running the Multiplex PCR program.

♦ Thermocycler should be outside the pre-PCR room ♦

- Prepare multiplex PCR master mix for pool 1 and pool 2 using the table below :

A	B	C	D
POOL 1	1x reaction volume (uL)	400x reaction volume (uL)	Notes
NEBNext Ultra II Q5 Hot Start 2x Master Mix (M0544L)	2.5	1115.00	master mix is made for 446 reactions to account for dead volume
Primer Pool 1 (10µM)	0.8	368.00	
Nuclease-free Water	0.2	92.00	
cDNA from step 3	1.5	-	
Total	5	1610	

A	B	C	D
POOL 2	1x reaction volume (uL)	400x reaction volume (ul)	Notes
NEBNext Ultra II Q5 Hot Start 2x Master Mix (M0544L)	2.5	1115.00	master mix is made for 446 reactions to account for dead volume
Primer Pool 2 (10µM)	0.8	368.00	
Nuclease-free Water	0.2	92.00	
cDNA from step 3	1.5	-	
Total	5	1610	

- Using a sptlabtech Dragonfly or similar liquid handler, dispense **3.5 µl** of the Pool 1 and Pool 2 PCR master mix into a 384 well plate labeled L-00X-POOL-1 and L-00X-POOL-2 respectively.
- Make sure to use different syringes and reservoirs for the Dragonfly runs. place the L-00X-POOL-1 and the L-00X-POOL-2 plates on ice.
- Using the Agilent Bravo or similar liquid handler, transfer **1.5 µl** of the cDNA from step 3 to the L-00X-POOL-1 and L-00X-POOL-2 plates respectively.
- Seal plates, spin down,improve and take plates to a thermocycler in the post-PCR lab space.
- Multiplex PCR program:

A	B
Program	Multiplex PCR
Lid	105°C
Volume	5µL

A	B	C	D
Steps	Temp	Time	Cycle
Step 1	98°C	30 sec	1
Step 2	98°C	15 sec	30*
Step 3	63°C	5 min	
Step 4	4°C	hold	1

*We found reducing the number of amplification cycles in the multiplex PCR reaction from 30 to 25 impacted the yield of genomes when processing plates that contained samples covering a broad range of Ct values similar to those commonly provided by our partners (refer fig. 3).

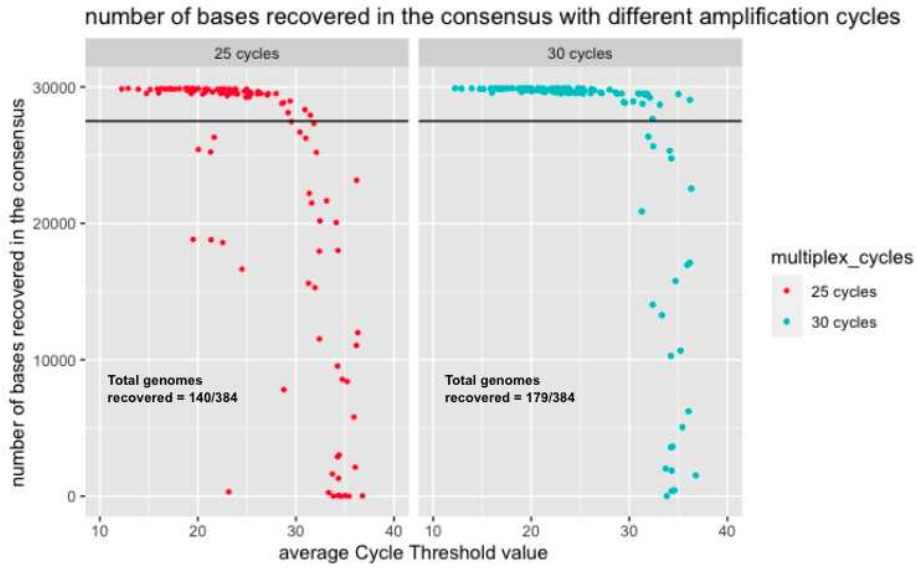
♦ Checkpoint: you can potentially store the multiplex PCR plate at -20C overnight. ♦

♦ Figures:

♦ Figure 3: Comparison of genome coverage with 25 and 30 cycles of PCR for samples across a wide range of Cts

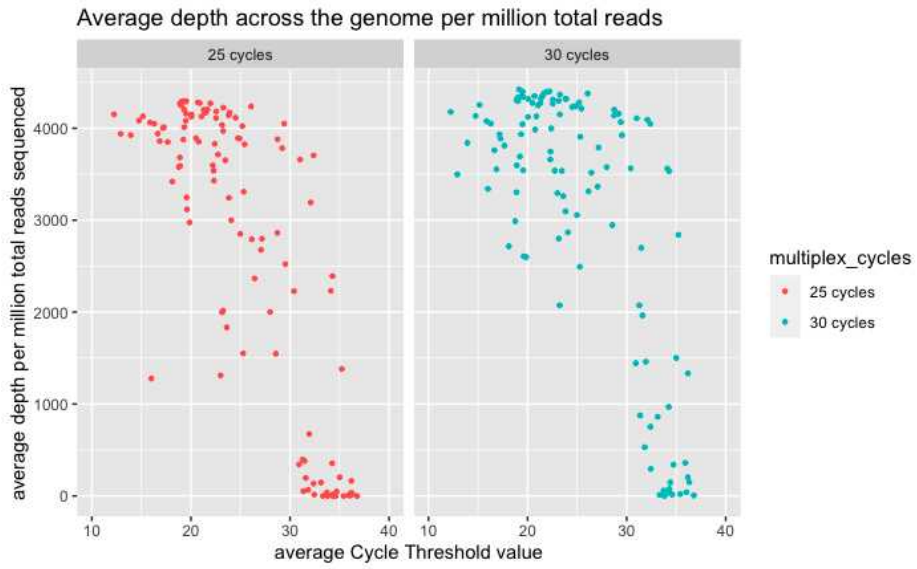
◊ Figure 3A:

.



30 cycles of amplification recovered 39 more genomes as compared to 25 cycles of amplification. This experiment was performed on the same set of 384 samples with different amplification cycles. All processes upstream and downstream of the multiplex PCR step were constant for both conditions.
Note: the black line (y-intercept) on the graph is set to 27500 bases, corresponding to 92% of SARS-CoV-2 genome sequence recovery, our in-house threshold for genome calling.

◊ Figure 3B:



30 cycles of amplification improve read depth for medium-high Ct samples. This experiment was performed on the same set of 384 samples with different amplification cycles. All processes upstream and downstream of the multiplex PCR step were constant for both conditions.

Multiplex - Pooling and Dilution of PCR products

5 1. Multiplex products are first pooled and then diluted 1:100 before tagmentation.

♦ Pooling:

- At this stage, amplicons are highly concentrated and can easily aerosolize causing cross-contamination. Before proceeding, centrifuge the multiplex plates (L-00X-POOL-1 and L-00X-POOL-2) for a few minutes. This will minimize potential contamination.

- Using Integra or similar liquid handler, transfer **2 µl** from source L-00X-POOL-1 plate to a fresh destination plate labeled as L-00X-POOL-1+2.

- Repeat the same **2 µl** transfer from L-00X-POOL-2 source plate to the same L-00X-POOL-1+2 destination plate. Do not mix using the Integra. Carefully seal the L-00X-POOL-1+2 destination plate, and spin down at high speed for 2 minutes. In the end, you should have **4 µl** to work with in the L-00X-POOL-1+2 plate.

♦ 1:100 Dilution:

Note: For the 1:100 dilution of pooled multiplex PCR reactions in L-00X-POOL-1+2 plate, we use an Echo Qualified 384-Well Polypropylene 2.0 Plus Microplate. (# PPL-0200) because it can hold more volume than the 384 Biorad HS PCR plate and allows us to perform a 1:100 dilution in a single step. Alternatively, you can do serial dilutions to achieve 1:100 dilution with Biorad HS PCR plates.

- Using an Integra or similar liquid handler, transfer **49.5 µl** of nuclease-free water to an Echo Qualified 384-Well Polypropylene 2.0 Plus Microplate. (# PPL-0200) destination plate. This can be pre-made and stored at 4°C.

- Using an Integra or similar liquid handler, transfer **0.5 µl** of from the L-00X-POOL-1+2 plate to the pre-made Echo Qualified 384-Well Polypropylene 2.0 Plus Microplate. (# PPL-0200) with **49.5 µl** of nuclease-free water in it. Label the plate as L-00X-POOL-1+2-1:100.

- Carefully seal and spin down the L-00X-POOL-1+2-1:100 plate.

- Transfer **0.5 µl** of the diluted pool from the L-00X-POOL-1+2-1:100 plate to a new Biorad HS 384 well PCR plate using an Integra or similar liquid handler. This transfer accounts for dead volume. The actual volume transferred is **0.4 µl** .

Tagmentation - Fragmentation and Adaptor ligation.

6 - CZBiohub uses a homebrew recombinant Tn5 transposase to fragment the DNA amplicons and ligate adapter sequences compatible with the Nextera dual index sequencing primers for Illumina sequencing. This protocol is modified from previously reported tagmentation-based protocols. [1,2,3]

- Pre-make a 5X stock solution of tagmentation buffer ("TAG"), and store at -20°C. Dilute to a final working concentration of 2.18X from 5X and aliquot in single-use Eppendorf tubes and store at -20°C.

- Recipe for 5X TAG buffer:

A	B
Tris-HCl, pH 7.4 (Lonza, 51247)	50 mM
MgCl2 (Invitrogen, AM9530G)	50 mM

- Make 1X Tn5 master mix using the following recipe:

A	B	C	D
	1x Reaction Volume (uL)	400X Reaction Volume (uL)	Notes
Amplicon DNA (1:100 dilution)	0.4		
TAG Buffer (From 2.18X stock)*	0.91428	512.00	master mix is made for 560 reactions to account for dead volume
N,N-dimethyformamide (DMF) (Sigma-Aldrich, 227056-1L)	0.08	44.80	
Homebrew Tn5 † (1:128)	0.20571	115.20	
Total	1.6	672.00	

* Note: final concentration of TAG Buffer is actually 1.2X.
† 1X Enzyme is calculated as the dilution of enzyme stock needed to achieve comparable activity to control Tn5 in the Nextera Kit using the same volume of input and the same volume of the enzyme. refer to tn5 batch testing for more details.

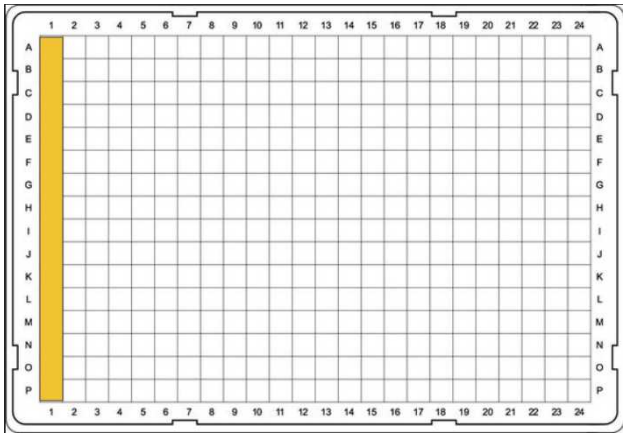
- more information to test homebrew Tn5 batch: **Comments on testing a Tn5 Batch (1).docx**

A	B
number of source wells	16
uL/well:	38.4
Pipette into wells A1-P1 of a new 384-well plate	

- Before making the master mix, make sure the thermocycler is running the Tagmentation program.
- In a chemical fume hood, mix DMF and TAG Buffer. Add homebrew recombinant Tn5 to the buffer mix. Move quickly to make the master mix.

NOTES: Tn5 is temperature-sensitive and can precipitate out of solution. Handle with care and avoid multiple freeze-thaw cycles. Stocks of homebrew recombinant Tn5 are diluted in a 50% glycerol mix. It is critical to slowly pipette up and down with a wide-bore or P1000 pipette to thoroughly mix the solution. DO NOT VORTEX.

- On ice, manually pipette **38.4 µl** of the Tn5 master Mix into wells A1-P1 of a Biorad HS 384-well PCR plate. [Seal and spin down briefly].
-



- With the sptlabtech Mosquito or similar liquid handler, use the Tn5 reaction mix plate as the source plate and the L-00X-POOL-1+2-1:100 plate from step 5 as the destination plate, transfer **1.2 µl** of Tn5 reaction mix to each well of the destination plate.

dimethylformamide

- Immediately seal the destination plate, spin down and place it on the thermocycler. Save the source plate to re-use in step 7.

Program	Tagmentation
Lid	105°C
Volume	2µL

A	B	C
Steps	Temp	Time
Step 1	55°C	5min
Step 2	4°C	hold

- While the Tagmentation program is running, start making the source plate for the tagmentation neutralization mixture. (you can use the same plate as the Tn5 source plate with different columns)

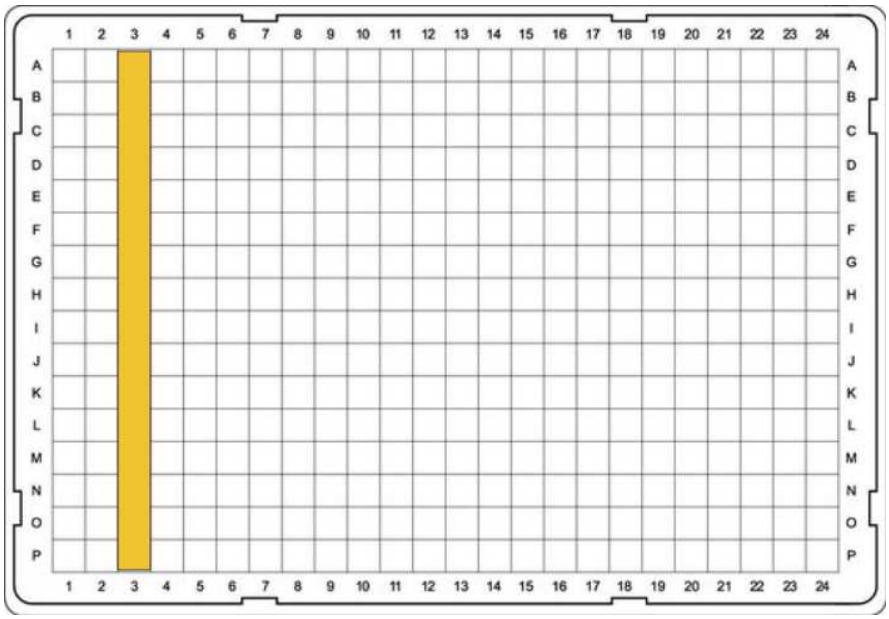
1. Tabula Muris Consortium, Shayan Hosseinzadeh2019.SmartSeq2 for HTP Generation of FACS Sorted Single Cell Libraries.**protocols.io** https://dx.doi.org/10.17504/protocols.io.2uwgexe
2. Hennig, B. P. et al. Large-Scale Low-Cost NGS Library Preparation Using a Robust Tn5 Purification and Tagmentation Protocol. G3 Genes Genomes Genetics 8, 79–89 (2018).
3. Picelli, S. et al. Tn5 transposase and tagmentation procedures for massively scaled sequencing projects. Genome Res 24, 2033–2040 (2014).

Neutralization

7
- This step is time-sensitive! Tn5 has some activity at room temperature, so move quickly to neutralize the Tn5 reactions.

- The Tn5 neutralization buffer corresponds to a 0.1% Sodium Dodecyl Sulfate (SDS, Fisher Scientific, BP166-500) solution. Prepare 1% SDS as a stock solution in advance and store at room temperature. Prepare single-use aliquots of the final 0.1% working solution in advance to streamline this step.

- Pipette the neutralization buffer into wells A3-P3 of the same Biorad HS 384-well PCR plate source plate used in step 6.



- Seal the plate, spin down briefly.
-
- With the mosquito use this plate as the source plate and the Tn5 reaction plate as the destination plate. Transfer **0.4 µl** of the neutralization solution to each well of the Tn5 reaction plate to start inactivation of the tagmentation reaction.

- Immediately seal the Tn5 reaction plate and centrifuge at full speed for 5 minutes.

Index PCR

8
- Using Dragonfly or similar liquid handlers dispense 3.5 ul of NEBNext Ultra II Q5 Master Mix-2X per well of a 384 well Biorad HS PCR plate. You can store this pre-aliquoted single-used plate at -20°C.
- Start Index PCR program on the thermocycler.
- Thaw a Nextera dual index barcode plate containing 5 µM stocks of unique i5 and i7 index primers in each well. Briefly spin down at maximum speed.
- Note: the Q5 plate should thaw quickly. Briefly spin down at maximum speed.
- Using an Integra or similar liquid handler, stamp 0.8µL from the dual barcode plate to your neutralized amplicon plate.
- Using the same tips, stamp 2.8 uL from the Q5 plate to the plate with neutralized and cleaned amplicons.

A	B
Reagents	1x Reaction Volume (uL)
Tagmented product from step 7	2
NEBNext Ultra II Q5 Master Mix (M0544L) (2X)	2.8
Nextera Dual Index Barcodes (i7 / i5) (5 uM)	0.8
Total per well	5.6

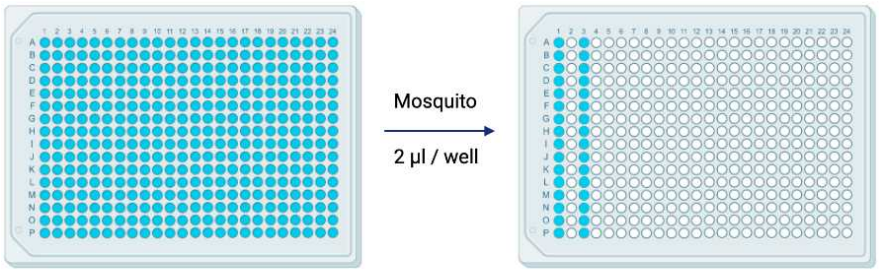
- Seal and spin all plates, making sure that there are no bubbles in the wells. Place the plate on the thermocycler.

Program	Index_PCR
Lid	105°C
Volume	4µL

A	B	C	D
	Temp	Time	Cycle
Step 1	72°C	3 min	1
Step 2	95°C	30 sec	1
Step 3	95°C	10 sec	8
Step 4	55°C	30 sec	
Step 5	72°C	1 min	
Step 6	72°C	5 min	1
Step 7	4°C	hold	1

Library pooling and SPRI cleanup

9
- Use mosquito or similar liquid handlers to pool **2 µl** of the library from each well of the library plate to A1 and A3 columns in a 384 well HS PCR plate.



created in BioRender.com

- Use a multichannel pipette to pool samples in the A1 and A3 columns to a clean sterile reservoir. Using a P1000 pipette aspirate the whole pooled volume to an Eppendorf tube.
- Use SPRIselect or homebrew SPRI beads to clean pooled library.
- Verify the total volume pooled and calculate the required volume of SPRI beads. Add room temperature SPRI beads at a ratio of 0.7X pooled library volume (SPRI beads volume: total pooled library volume) to the PCR enriched/barcoded library reaction. Mix well.
- Incubate for 5 minutes at room temperature.
- Place the Eppendorf tube on a magnetic rack and incubate for 5 mins.
- Remove supernatant. Add **700 µl** of 80% ethanol to the Eppendorf tube while on the magnetic rack. Incubate at room temperature for 1 min then discard the supernatant.
- Repeat the ethanol wash step a total of 2 times.
- Dry the beads for 5 mins while on the magnetic rack.
- Remove the Eppendorf tube from the magnetic rack. Elute DNA from beads into **53 µl** of Nuclease free water.
- Vortex to mix. Spin tubes and incubate for 5 mins at room temperature off the magnetic rack.
- Place the Eppendorf tube back on the magnetic rack until the solution is clear ~ 5 mins.
- Remove **50 µl** of the supernatant and transfer to a clean Eppendorf tube.
- Perform Quality control on the clean library using TapeStation or Bioanalyzer.

Quality control

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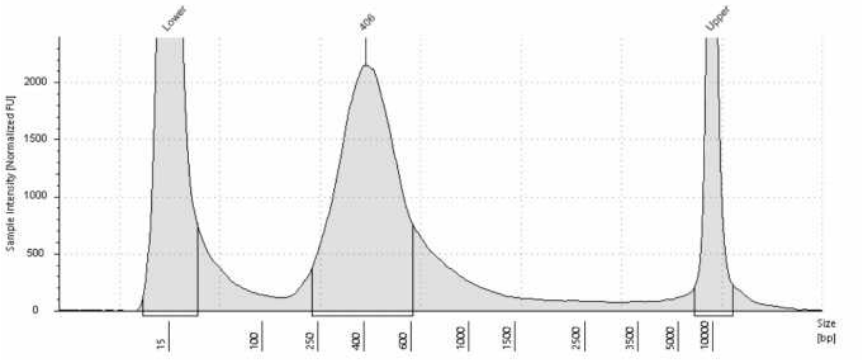
- Quantify library using a fragment analyzer such as TapeStation or BioAnalyzer.
- Dilute the final library appropriately to fall in the detection range of the assay (HS D5000, D5000, Bioanalyzer). To do this perform upfront quantification of the cleaned library using Qubit ds DNA assay or similar.
- For Agilent D5000 Tapestation assays dilute the final library 1:10 and 1:100.
- Run undiluted, 1:10, and 1:100 as per the assay guidelines on Agilent Tapestation.
- Tapestation/Bioanalyzer gives important information about primer dimers which are seen around 140 bp. Library peak size should be around 400 bp. additional SPRI cleanup might be necessary to get rid of primer dimers. The clean amplicon library should look as follows.



Tapestation output showing major library peak detectable at 400 bp. There are no detectable 140 bp primer dimers observed in the library.

- For in-house applications, we measure the region concentration using Agilent tapestation. Region concentration is calculated using the area within 200 bp to 1000 bp markers.
- From the past 5 library preps, the average region concentration or yield of the library was 38 nmol/L (range: 20.8 - 52.8 nmol/L), and the average library size was 452 bps (range: 425-473 bps).
- Given samples with a broad range of Ct values (12-30) in a typical library prep, we empirically determined that sequencing 1 million total reads per sample maximized genome recovery yield from a 2 x 150 bp paired-end sequencing run on a Novaseq 6000, Nextseq 500, or Nextseq 2000.