Upload image

Jul 28, 2020

ARTIC-NEB: SARS-CoV-2 Library Prep

Lusajo Mwakibete[1], Cristina Tato[2], Vida Ahyong[1], Amy Kistler[1], Manu Vanaerschot[1], Angela Detweiler[1], Michelle Tan[1]

[1]CZ Biohub; [2]Chan Zuckerberg Biohub

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

Lusajo Mwakibete
CZ Biohub

ABSTRACT

This experiment was carried out to investigate the effect of fragmentation time on the genome recovery of SARS-COV2 from COVID-19 positive patient samples using an iSeq. The experiment uses the ARTIC protocol to attain the amplicon from the extracted RNA, followed by FS DNA Library Prep Kit to make the amplicon library. We investigated the fragmentation time at the beginning of the FS DNA library prep. Fragmentation time of 5 minutes gave the best genome coverage having the least dropout regions.

The ARTIC protocol was adapted from Josh Quick 2020.nCoV-2019 sequencing protocol.**protocols.io**dx.doi.org/10.17504/protocols.io.bdp7i5rn

PROTOCOL CITATION

Lusajo Mwakibete, Cristina Tato, Vida Ahyong, Amy Kistler, Manu Vanaerschot, Angela Detweiler, Michelle Tan 2020. ARTIC-NEB: SARS-CoV-2 Library Prep. **protocols.io** https://protocols.io/view/artic-neb-sars-cov-2-library-prep-bh3cj8iw

CREATED

Jun 30, 2020

LAST MODIFIED

Jul 28, 2020

PROTOCOL INTEGER ID

38724

GUIDELINES

The following protocol is updated from the https://www.biorxiv.org/content/10.1101/2020.05.11.088724v1.full.pdf protocol to the amplicon multiplexing step. Then it follows the **NEBNext Ultra II FS DNA Library Prep Kit protocol.**

MATERIALS

NAME	CATALOG #	VENDOR
Qubit® dsDNA HS assay kit, 100 reactions	Q32851	Life Technologies

Dilution of Input Viral RNA

1 **Caution!** Carryout the following dilution steps with DNAse/RNAse free water in PCR-pre, RNAse free environment (Biosafety cabinet).
RNA was extracted using the Zymo *Quick* DNA/RNA Viral MagBead Kit (R2141).
RT-PCR CT values dictate the viral RNA input as following:

12-15	1:100
15-18	1:10

18-35	no dilution

cDNA

2 Random Hexamer Priming

Set up the following reaction (8-Strip Tube).

	1 rxn vol stock (µL)	0.5 rxn vol stock (µL)
60µM Random hexamers and anchored polyT(23) (NEB S1330S)	1	0.5
10 mM dNTPS (NEB N0447)	1	0.5
RNA Input Sample	11	5.5
Total Volume	13	6.5
	µL/rxn	**µL/rxn**

Mix the mixture with a pipette thoroughly, place the reaction in the thermocycler with the following setting:

	Temperature	Time
Step 1	65°C	5 min
Step 2	4°C	Hold
Lid	105°C	

3 Reverse Transciptase (RT)

Make the following master mix and add to the Random Primed RNA

	1 rxn vol stock (µL)	0.5 rxn vol stock (µL)
SuperScript IV 5X Buffer	4	2
100mM DTT	1	0.5
RNase OUT RNAse Inhibitor	1	0.5
Superscript IV Reverse Transcriptase	1	0.5
Total Volume	7	3.5
	µL/rxn	**µL/rxn**

Mix the mixture with a pipette thoroughly, place the reaction in the thermocycler with the following setting:

	Temperature	Time
Step 1	25°C	5min
Step 2	42°C	50min
Step 3	70°C	10min
Step 4	4°C	hold
Lid	105°C	

◊ checkpoint: can freeze overnight (-20°C)◊

cDNA Amplicon Amplification

4 MULTIPLEX PCR

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

Lyophilized primers were resuspended to a concentration of 100 uM.

Primers were pooled in "Pool 1" or "Pool 2" according to ARTIC protocol [1]. 100 uM primer pools were further diluted to 10uM. 10µM primers can be pre-aliquoted and stored at -20°C.
The table below was used to prepare separate master mixes for Pool 1 and Pool 2 primers respectively.

	1 rxn vol stock (µL)
NEBNext Ultra II Q5 Hot Start 2x master mix	12.5
Primer pool at 10 µM (1 or 2)	4
Nuclease-free water	6
cDNA from Step 3	2.5
Total Volume	25
	µL/rxn

Mix the mixture with a pipette thoroughly, place the reaction in the thermocycler with the following setting:

	Temperature	Time	Cycle
Step 1	98°C	30 sec	
Step 2	98°C	15 sec	30*
	63°C	5 min	
Step 3	4°C	Hold	
Lid	105°C		

*Number of cycles depends on the input RNA Ct values. 30 cycles works for all Ct values, lower sample Ct values less cycles required.

5 Pool and SPRI (Magnetic Bead Purification) Clean

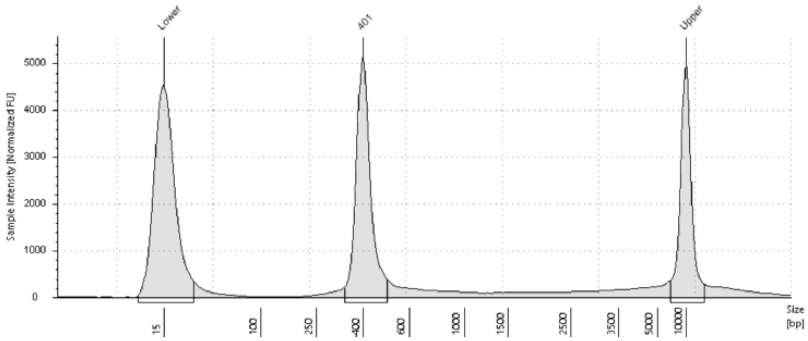
Pool multiplexed samples from pool 1 and pool 2 together for each respective sample.
*allow beads to sit in RT for 30 mins prior

1. Combine Pool 1 and Pool 2 PCR reactions together from the prior multiplex PCR step.
2. Use *SPRI bead* 1x ratio of beads-to-total volume of sample. Prep 80% EtOH.
3. Add 50µL of room temperature beads (1x) to the pooled PCR reaction (50µL). Mix well.
4. Pulse spin the tubes, but be sure not to spin down beads. Incubate for 5 mins at room temperature.
5. Place samples on magnetic rack, and incubate for 5 mins on the rack.
6. Remove supernatant.
7. Add 200µL of 80% EtOH to samples while on the magnetic rack. Incubate at room temperature for 1 min then remove the supernatant.
8. Repeat EtOH wash step for total of 2 times.
9. Air dry the beads for 5 mins while on the magnetic rack.
10. Remove tube from magnetic rack. Elute DNA from beads into 69µL of 0.1x TE Buffer, 10mM Tris-HCl, or Nuclease free water.
11. Vortex to mix. Spin tubes and incubate for 5 mins at room temperature off the magnetic rack.
12. Place on magnetic rack until solution is clear ~ 5 mins.
13. Remove 67µL of the supernatant and transfer to a clean nuclease free PCR tube.

◊ **checkpoint: can freeze overnight (-20°C)**◊

6 Quantification of Amplicon

Quantify amplicon using a fragment analyzer such as TapeStation and BioAnalyzer. Use Qubit dsDNA High Sensitivity (HS) assay to get amplicon concentration prior to loading sample into fragment analyzer, to make sure you don't overload the ScreenTape/Chip. The concentration quantification aids with selecting DNA input for the **NEBNext Ultra II FS DNA Library Prep** which follows.



A peak should be spotted at 400bp which coincides to the size of the amplified amplicons of the SARS-COV-2 genome.

Amplicon Library Prep

7 Using FS DNA Library Kit (E7805,E6177) to finish amplicon libraries. Make sure the input Amplicon concentration is between 10 -100ng.

Fragmentation

Make the following master mix (The reaction has been scaled down to 0.5X from the normal protocol FS DNA Library Prep Kit.

	0.5X rxn vol stock (µL)
DNA input (10-100ng)	13
NEBNext Ultra II FS Reaction Buffer (Yellow)	3.5
NEBNext Ultra II FS Enzyme Mix (Yellow)	1
Total Volume	17.5

Mix the mixture with a pipette thoroughly, place the reaction in the thermocycler with the following setting:

	Temperature	Time
Step 1 (Fragmentation)	37°C	5 min
Step 2 (Enzyme Deactivation)	65°C	30 min
Step 3	4°C	Hold
Lid	75°C	

8 **Adaptor Ligation**

Add the following components directly to the prior FS reaction mixture.

	0.5X rxn vol stock (µL)
FS Reaction Mixture	17.5
NEBNext Ultra II Ligation Master Mix* (Red)	15
NEBNext Ligation Enhancer (Red)	0.5
NEBNext Adaptor for Illumina (1:100 dilution)	1.25
Total Volume	34.25

*add adaptor separately from the master mix to avoid adapter dimers.
Mix the mixture with a pipette thoroughly, place the reaction in the thermocycler with the following setting:

	Temperature	Time
Step 1	20°C	15 min
Step 2	4°C	Hold
Lid	Off	

9 SPRI (Magnetic Bead Purification) Clean

*allow beads to sit in RT for 30 mins prior

1. Use *SPRI bead* 0.9x ratio of beads-to-total volume of sample. Prep 80% EtOH.
2. Add 30.83µL of room temperature beads (0.9x) to Adaptor Ligation reaction (34.25µL). Mix well.
3. Pulse spin the tubes, but be sure not to spin down beads. Incubate for 5 mins at room temperature.
4. Place samples on magnetic rack, and incubate for 5 mins on the rack.
5. Remove supernatant.
6. Add 200µL of 80% EtOH to samples while on the magnetic rack. Incubate at room temperature for 1 min then remove the supernatant.
7. Repeat EtOH wash step for total of 2 times.
8. Air dry the beads for 5 mins while on the magnetic rack.
9. Remove tube from magnetic rack. Elute DNA from beads into 17µL of 0.1x TE Buffer, 10mM Tris-HCl, or Nuclease free water.
10. Vortex to mix. Spin tubes and incubate for 5 mins at room temperature off the magnetic rack.
11. Place on magnetic rack until solution is clear ~ 5 mins.
12. Remove 15µL of the supernatant and transfer to a clean nuclease free PCR tube.

◊ **checkpoint: can freeze overnight (-20°C)**◊

10 PCR ENRICHMENT/BARCODING

	0.5X rxn vol stock (µL)
Purified, adaptor-ligated cDNA	7.5
USER Enzyme (Cat no. M5505L, 250uL)	1.5
NEBNext Ultra II Q5 Master Mix (Blue)	12.5
i7 barcoded primer (NEB index primer/TruSeq/or similar)*	2.5
i5 barcoded primer (NEB Universal primer/TruSeq/or similar)*	2.5
Total volume	26.5

*The i7 and i5 primers can be premixed, each primer should be 5µM in the Primer Mix

Mix the mixture with a pipette thoroughly, place the reaction in the thermocycler with the following setting:

	Temperature	**Time**	**Cycle**
Step 1	37°C	15 min	1
Step 2	98°C	30 sec	1
Step 3	98°C	30 sec	12
	65°C	75 sec	
Step 4	65°C	5 min	1
Step 5	4°C	Hold	

* depending on the RNA input number of cycles can be varied accordingly. (I.E. High cDNA amplicon input, less cycles and vice-versa).

11 SPRI (Magnetic Bead Purification) Clean

*allow beads to sit in RT for 30 mins prior

1. Use *SPRI bead* 0.8x ratio of beads-to-total volume of sample. Prep 80% EtOH.
2. Add 42.4µL of room temperature beads (0.9x) to the PCR enriched/barcoded library reaction (53µL). Mix well.
3. Pulse spin the tubes, but be sure not to spin down beads. Incubate for 5 mins at room temperature.
4. Place samples on magnetic rack, and incubate for 5 mins on the rack.
5. Remove supernatant.
6. Add 200µL of 80% EtOH to samples while on the magnetic rack. Incubate at room temperature for 1 min then remove the supernatant.
7. Repeat EtOH wash step for total of 2 times.
8. Air dry the beads for 5 mins while on the magnetic rack.
9. Remove tube from magnetic rack. Elute DNA from beads into 27µL of 0.1x TE Buffer, 10mM Tris-HCl, or Nuclease free water.
10. Vortex to mix. Spin tubes and incubate for 5 mins at room temperature off the magnetic rack.

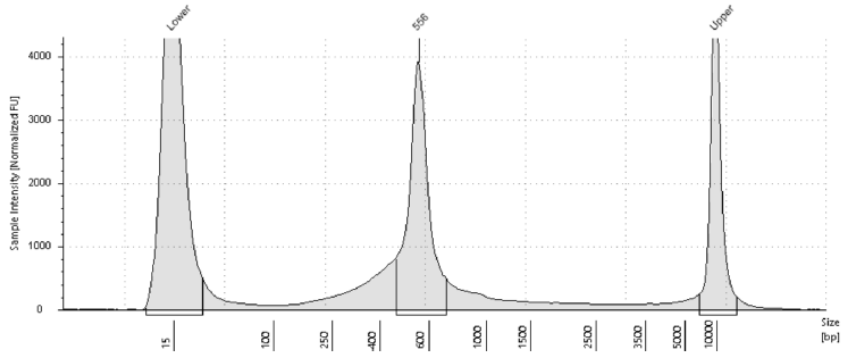
11. Place on magnetic rack until solution is clear ~ 5 mins.
12. Remove 25µL of the supernatant and transfer to a clean nuclease free PCR tube.

◊ **checkpoint: can freeze overnight (-20°C)**◊

Quality Control of Amplicon Library

12 Quantification of Amplicon Library

Quantify library using a fragment analyzer such as TapeStation and BioAnalyzer. Use DNA HS Qubit to get library concentration prior to loading sample into fragment analyzer, to make sure you don't overload the machine. The profile of the library will aid in showing if there are any contaminants in your library such as adapter dimers at around 140bp. In addition, more SPRI clean-ups might be required if adapter dimers are present in the library. The clean amplicon library should look as following.



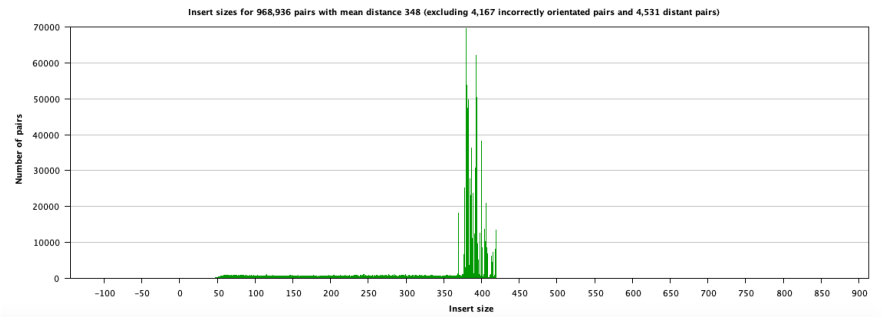
Tapestation electropherogram showing amplicon library (post-spri clean up) with a peak around 556bp. The amplicon average peak size should be around 450-500bp(400bp amplicon plus 140bp adaptors, need to take into account the fragmentated amplicon insert sizes shorter than 400bp). Additional SPRI bead cleanups might be required to get rid of adaptor dimers if present.

Analysis

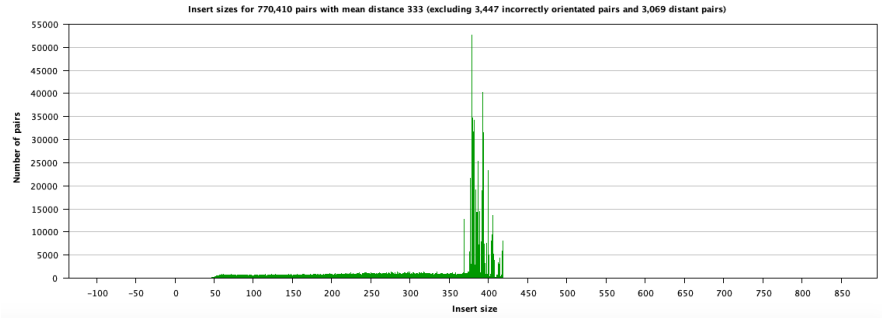
13 Analysis of Fragmentation Time :

Insert Size:

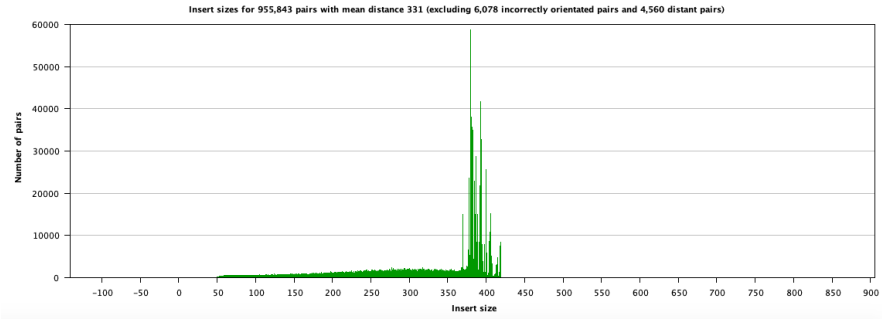
The fragmentation time varied the insert sizes for our libraries. 5 mins and 10 mins fragmentation times decreased a majority of our insert size from around 400bp to 200-250bp. This is good for individuals who want to sequence the SARS-CoV-2 genome with an ISEQ whilst using the artic protocol. This will allow the 2 by 150 reads, to attain better reads spanning the entire genome, with the shorter fragment sizes. Thus we suggest the fragmentation time of 5 mins instead of 10 minutes because we don't want to overfragment our amplicon inserts.



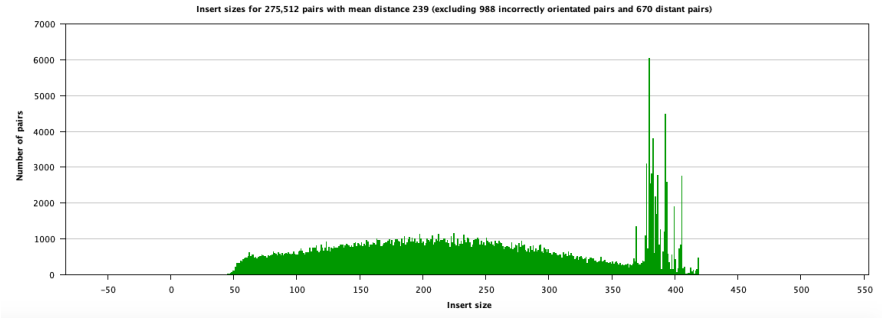
0 Min Fragmentation



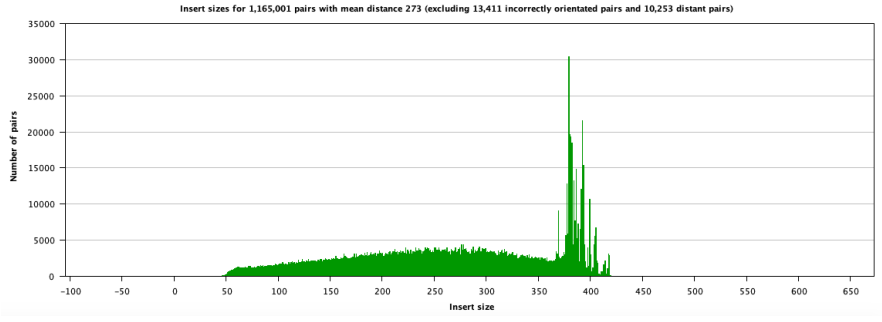
1 Min Fragmentation



3 Min Fragmentation

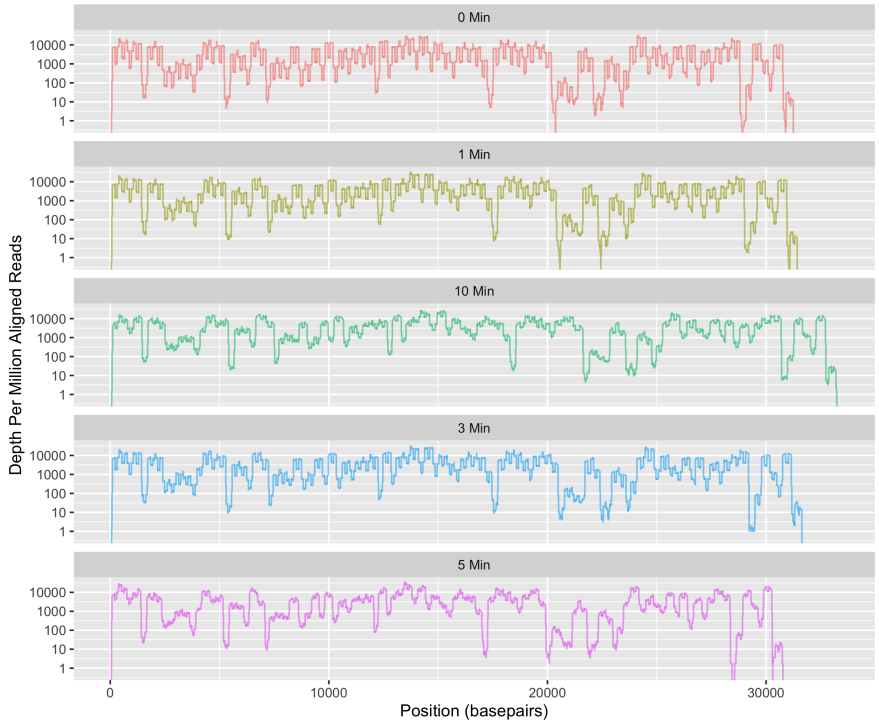


5 Min Fragmentation



10 Min Fragmentation

Depth Coverage:



Graph to show coverage depth per million vs position on varying fragmentation time. The title (e.g. Five, One) is fragmentation time in minutes at the beginning of building the cDNA amplicon library (Step 7). Depth Per Million Aligned Reads calculated as following Depth = (Raw Coverage Per Respective Position/ Total Aligned Reads) * 10^6. Y-axis (log-scale)