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Life Sciences

Increasing SARS-CoV-2 sequencing throughput by leveraging a combination of tip and acoustic liquid handlers.

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Highlights:

- **High throughput sample preparation:** Combined use of the Biomek i7 hybrid and Echo 525 liquid handlers allows for an efficient, accurate, accelerated workflow which significantly increases output.
- **Minimize tip usage:** Use of the Echo 525 Liquid Handler (LH) makes it possible to complete the library preparation using only 2 tips per sample.
- **Faster turnaround:** Sample preparation can be completed in under 8 hours with a single pooled bead cleanup for 384 samples. We modified the previously published 275 bp method to use paired-end 150 base sequencing runs, which allows faster sequencing.

Abstract

Higher throughput SARS-CoV-2 sequencing methods are required to surveil the spread of variants. The ideal protocol is low cost, has a simple workflow, is high throughput, and compatible with a wide variety of sequencing instruments. While a variety of methods have been developed none meets all of these criteria. In this Application Note, we describe a method we developed and deployed on the Beckman tip-based and acoustic liquid handling platforms to process samples in batches of 384 samples, which can be completed in a single shift, and uses only two tips per sample.

Introduction

Genomic surveillance of the SARS-CoV-2 virus is critical to pandemic response for two reasons. First, in order to detect variants of concern, it is estimated that a minimum of 5% of positive samples must be sequenced. Globally, this can mean tens of thousands of samples per day. Second, early detection is critical to prevent the spread of concerning variants. In this Application note, we demonstrate how Echo acoustic liquid handling combined with Biomek tip-based liquid handling and amplicon-only sample preparation methods can greatly increase the throughput and lower tip usage for SARS-CoV-2 sequencing.

The ARTIC network has released several protocols adopted by the global sequencing community[1]. A widely used tailed protocol based on 400 bp amplicons is low cost and high throughput on the preparation side but it requires paird end 250 base sequencing reads[2]. This limits the samples to the few Illumina kits on the MiSeq or NovaSeq that can take two days to complete. Additionally, this tailed method requires four separate PCR pools per sample instead of the two pools for the traditional ARTIC methods. To address these challenges, we have developed a tailed 275 bp method that uses two PCR pools per sample (Figure 1).

Methods

We developed a modified tailed library prep method based on a 275 bp ARTIC SARS-CoV-2 protocol[3, 4]. Total nucleic acid or viral RNA fragments were extracted from positive SARS-CoV-2 samples.

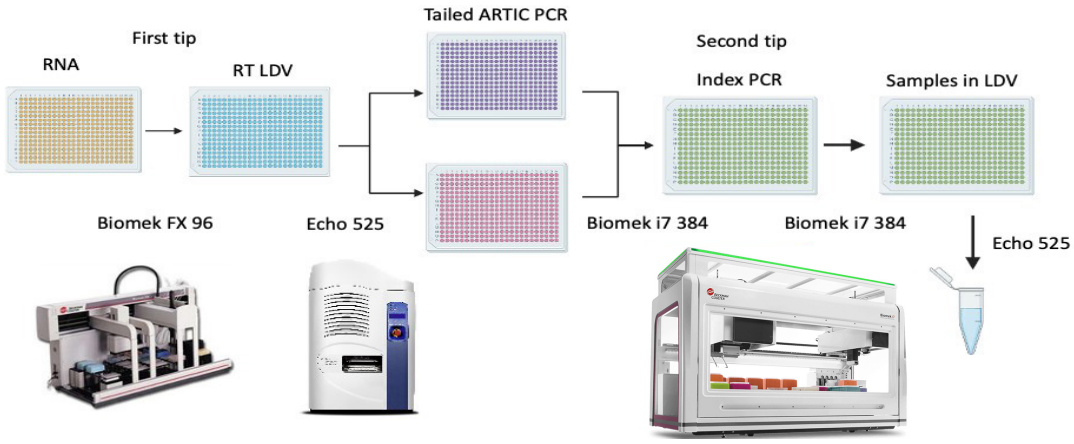


Figure 1. Sample preparation workflow utilizing Biomek FX, Biomek i7 hybrid and Echo 525 liquid handlers.

cDNA generation

8 µL of RNA from four 96-well plates are transferred into a 384-well Echo LDV plate containing 2 µL NEB LunaScript RT SuperMix using the 96-channel head on a Biomek FX (Figure 1 and 2A). After mixing and centrifuging, reverse transcription is performed on a flat block thermocycler (Bio-Rad C1000 384-well with a thermal pad and shim) for 2 minutes at 25°C, 20 minutes at 55°C, 1 minute at 95°C, and 4°C hold.

cDNA amplification

A set of tailed 275 bp amplicon primer pools was created based on a non-tailed design[4]. A master mix for each primer set was created according to Table 1 and aliquoted into 384-well Bio-Rad PCR plates.

Multiplexed master mix	Volume (µL)
NEBNext Ultrall Q5 master mix	5
Pool 1 or Pool 2 primer mix (10µM)	1.6
Nuclease-free water	2.4
Total	9

Table 1. The reagent volumes in the amplification reaction.

1 µL of amplified cDNA was transferred with the Echo 525 LH. Plates were sealed, vortexed, and spun down. Samples were amplified with the following parameters: 98°C 30 s, 98°C 15 s, 63°C 5 mins, for 35 cycles, hold at 4°C. If desired, the cDNA can be run on an electrophoresis instrument. Products should be about 300 bp in size (Figure 3A).

Indexing PCR

An indexing PCR plate was created according to Table 2 and transferred into a Bio-Rad 384-well plate.

Indexing PCR Mix	Volume (µL)
NEBNext Ultrall Q5 master mix	5
5µM TruSeq i7/i5 Indexing primer mix	2
Water	2
Total	9

Table 2. The reagent volumes in the indexing PCR.

ALPs maintained at -15 ˚C

The Biomek i7 was used to pool and create a 1:100 dilution of the amplified cDNA. 1 µL of the diluted cDNA pool was transferred into the indexing PCR plate (Figure 1 and 2B). After sealing, mixing, and centrifugation, the plate was thermocycled with the following parameters: 98°C 30 s, 98°C 15 s, 65°C 1 min 15 s, for a total of 7 cycles, 65°C 5 mins, hold at 4°C.

Sample pooling and cleanup

After the indexing PCR, samples were transferred with the Biomek i7 workstation into an Echo LDV plate using the same tip from the last step. 0.5 µL of each sample is pooled with the Echo 525 LH into a 384-well Bio-Rad PCR plate. The pooled samples are transferred into a microtube and 0.8x volume of AMPure XP beads is mixed with the sample pool. After placing the tube on a magnet, the supernatant is removed by pipetting. While the tube is on the magnet, two 70% ethanol washes are performed. After 5 minutes of drying, the beads are suspended for 30 s. The tube is placed on a magnet and the eluate containing the library is transferred to a new tube.

QC and sequencing

Samples are checked with an Agilent TapeStation or BioAnalyzer. The sample should result in a library around 400 bp (Figure 3B). A final quantitation was performed on the pool by digital PCR. Each pool of 384 samples is sequenced on an Illumina NovaSeq SP 300 lane, generating on average over 1 million reads per sample. After sequencing, samples were analyzed with IDseq[5]. In brief, IDseq aligns reads against the reference genome, trims primer sequences, calls variants, and calculates coverage.

Figure 2. Deck layouts for Biomek FX and Biomek i7 hybrid workstation protocols. **A)** FX transfers from 96 well plate to separate quadrants of 384 RNA aliquot and RT plate. **B)** i7 hybrid ARTIC pooling, diluting, and transfer to index plate.

Results and Discussion

Many groups have employed ARTIC methods due to speed and cost but each has its own limitations. The original ARTIC method used primer pools to amplify 400 bp fragments of the SARS-CoV-2 genome. These fragments undergo a cleanup and traditional library preparation (fragmentation, addition of adapters, and PCR amplification). A newer method, the Tailed ARTIC approach is higher throughput on the preparation side. It appends Illumina Nextera adapter sequences on the ARTIC primers. This allows completion of libraries with a second indexing PCR reaction instead of a traditional library prep. The two downsides to this method are that the multiplexed amplification is split across 4 pools instead of 2 and the libraries must be sequenced on paired-end 250 base runs, limiting the types of sequencers it can be run on.

We performed a 400 bp tailed ARTIC workflow which required two multiplexed PCRs per sample and produced final libraries around 500 bp. This approach limited sequencing to Illumina MiSeq and NovaSeq SP kits only and had a sequencing time of 2 days which was not optimal for throughput. The ideal method to ensure the surveillance of positive COVID samples requires high throughput, low cost, and rapid turnaround time. During the plastics shortage brought about by the pandemic reduced tip usage was also required.

Our group decided to optimize the Tailed ARTIC approach to deploy it in a high throughput manner that minimizes tips to two per sample to deal with current supply chain challenges by leveraging tip-based and acoustic liquid handling. We developed a 275 bp tailed ARTIC workflow with two multiplexed PCRs per sample with a single bead cleanup step that can be completed in a workday. We automated this method with a combination of tip and acoustic liquid handling systems to process batches of 768 samples using only two tips per sample. This new approach uses paired-end 150 base sequencing runs which allows sequencing on any Illumina platform.

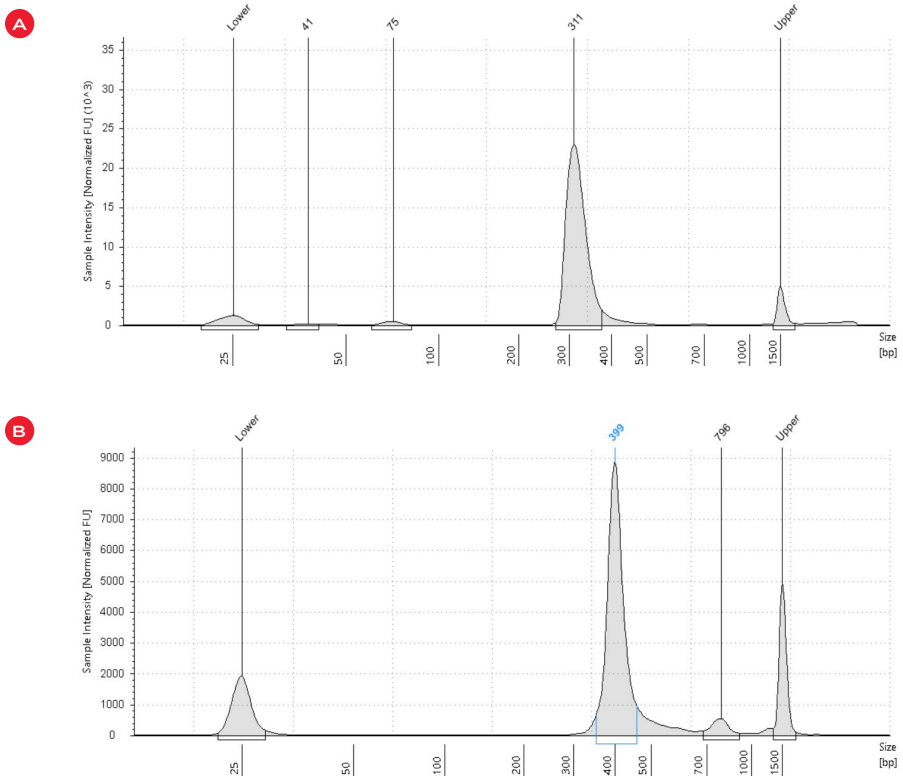
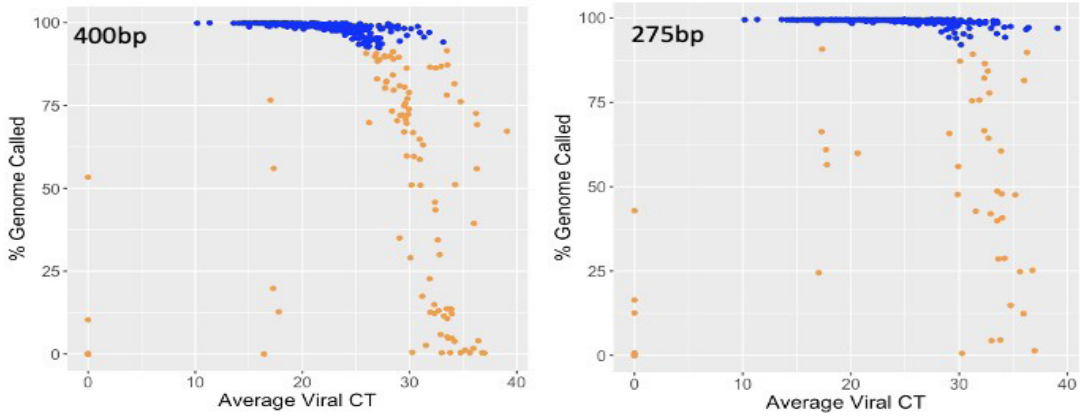


Figure 3. TapeStation traces of amplified cDNA **(A)** and final libraries for sequencing **(B)**.

We used an Illumina NovaSeq6000 to run 384 barcoded libraries per lane of an SP 300 flow cell averaging over 1 million reads per sample. We analyzed our demultiplexed data with IDseq which has our primer sequences to ensure proper trimming of sequences. Representative coverage plots of samples show good coverage across a range of sample Cts and more even coverage in low Ct samples (Figure 4). Samples with Ct less than 30 typically have a higher pass rate of greater than 92% genome called. The 275 bp method performs better with Ct values greater than 25 when compared to our tailed 400 bp method (Figure 5). We hypothesize higher Ct samples could be more degraded and better suited for shorter amplicons in the 275 bp method.

Figure 4. Low, mid and high Ct valued samples with greater than 92% genome coverage.



Ct distribution	Ct = 0	Ct < 15	Ct 15-20	Ct 20-25	Ct 25-30	Ct 30-35	Ct 35-40
Number of samples in Ct range	12	8	84	120	101	48	11
275 bp pass rate	0%	100%	94%	99%	97%	52%	36%
400 bp pass rate	0%	100%	94%	100%	61%	13%	0%

Figure 5. Sample pass rates using the tailed 400 bp or tailed 275 bp method. Plots show sample Ct vs % genome called after processing in IDSeq. Samples passing the cutoff are blue while failed samples are orange. The table lists the number of samples in each Ct bin and the percent of samples that passed in each bin.

Summary

Utilizing both the Echo acoustic LH and the tip-based Biomek i7 Hybrid workstation with this new protocol enables our group to process several 384-well plates of SARS-CoV-2 samples per day, greatly increasing our throughput and reducing staffing requirements. Our method uses paired-end 150 base sequencing runs, allowing faster sequencing compared to the previously published protocol. The additional benefit of using Echo 525 LH is the reduction in pipet tips which are currently in short supply across the industry. In our current protocol, we have been able to run the process from RNA to NGS library with only two tips per sample. In future directions, we hope to further leverage the small volume transfers of the Echo 525 LH and cut current 10 µL reaction volumes to further decrease costs. Although we used three liquid handling systems, this workflow can be executed With an Echo 525 LH and a single Biomek i7 LH with dual 96 and 384 well heads.

References

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3. Emma Betteridge, Naomi Park, Keith James, Jillian Durham, Josh Quick 2020. COVID-19 ARTIC v3 Illumina library construction and sequencing protocol - short amplicons (275bp). Protocols.io **https://dx.doi.org/10.17504/protocols.io.bh4zj8x6**

4. Delsy Martinez, Tyler Miyasaki, Eric Chow 2021. UCSF CAT COVID-19 Tailed 275bp v3 ARTIC protocol v1. Protocols.io **https://protocols.io/view/ucsf-cat-covid-19-tailed-275bp-v3-artic-protocol-v-bsfknbkw**

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Materials

Equipment	Manufacturer
Biomek i7 Automated Workstation	Beckman Coulter Life Sciences
Allegra 6 Microplate Centrifuge	Beckman Coulter Life Sciences
Biomek FX LH	Beckman Coulter Life Sciences
Echo 525 LH	Beckman Coulter Life Sciences
GeneAmp PCR System 9700	Applied Biosystems
C1000 Touch Thermal Cycler	Bio-Rad
TapeStation 4200	Agilent Technologies
NovaSeq 6000	Illumina

Table 1. Instruments used

Reagents	Manufacturer	Part Number
LunaScript RT SuperMix kit	New England BioLabs	E3010L
NEBNext Ultra II Q5 Master Mix	New England BioLabs	M0554L
Agencourt AMPure XP beads	Beckman Coulter Life Sciences	A63880
D1000 Sample Buffer Reagents	Agilent Technologies	5067-5583

Table 2. Reagents used

Consumables	#	Manufacturer	Part Number
384-Well Low Dead Volume Microplate, Echo Qualified	2	Beckman Coulter Life Sciences	001-12782
384-well polypropylene Source Microplate, Echo Qualified	1	Beckman Coulter Life Sciences	001-14622
384 Hard-Shell PCR MicroPlate, 384 well, thin wall	4	Bio-Rad	HSP-3805
Microseal 'B' seal Seals	7	Bio-Rad	MSB1001
96-well Tips 30 µL	4	Beckman Coulter Life Sciences	C62991
Biomek P50 Pipettte Tips	1	Beckman Coulter Life Sciences	A21586
Biomek i-Series, 40 µL pipette, sterile, filtered	1	Beckman Coulter Life Sciences	B85760
D1000 ScreenTape	1	Agilent Technologies	5067-5582
NovaSeq XP 2-Lane Kit v1.5	1	Illumina	20043130
NovaSeq 6000 SP Reagent Kit v1.5 (300 cycles)	1	Illumina	20028400

Table 3. Consumables per run

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