Version 1

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UCSF CAT COVID-19 Tailed 275bp v3 ARTIC protocol v1 V.1

Forked from COVID-19 ARTIC v3 Illumina library construction and sequencing protocol - short amplicons (275bp)

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

Coronavirus Method Development Community

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ABSTRACT

This is a tailed amplicon method similar to https://www.protocols.io/view/sars-cov-2-tailed-amplicon-illumina-sequencing-bipikdke but based on the shorter 275nt amplicons in https://www.protocols.io/view/covid-19-artic-v3-illumina-library-construction-an-bh4zj8x6. One modification from the Gohl trailed protocol is the use of partial TruSeq tails instead of Nextera tails and using two vs four multiplexed pools. The advantage of this method is the speed and low cost due to several reasons.

1. Only two separate cDNA ARTIC PCR reactions are required.
2. After the indexing PCR step using TruSeq indexing primers, equal volumes are pooled and a single tube bead cleanup is performed.
3. These libraries only require PE150 sequencing.

The workflow can be completed in under 8 hour. If a small number of samples are prepared, a MiSeq Nano or MIcro run can be used, for a total time from RNA to FASTQs in roughly 24 hours.

Note that this primer design has a 15bp primer overlap region around 20.5kb that results in 15 bases that cannot be sequenced with this method. We are investigating other primers in this region to recover these bases.

We have optimized this method to process samples in 384-well format using a Beckman Biomek i7 and a Labcyte Echo to minimize tip usage (three tips per sample). The procedure can also be performed manually at lower scale or in 96-well format, but you may need to increase the volume of the reactions for more accurate pipeting.

A cost effective manner to purchase this oligo set is with IDT oPools. At list price, you can order the two pools for a roughly $200 each. This is enough to prepare 500 samples at our volumes. You can also list each oligo two in a pool to get twice the amount of oligo at twice the cost.

PROTOCOL CITATION

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

FORK NOTE

FORK FROM

Forked from COVID-19 ARTIC v3 Illumina library construction and sequencing protocol - short amplicons (275bp), Naomi Park

KEYWORDS

COVID-19, SARS-Cov-2, amplicon sequencing, ARTIC, Illumina library construction, coronavirus

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GUIDELINES

It is vital cDNA setup is performed in a laboratory in which post-PCR COVID-19 amplicons are not present, to minimize any risk of sample contamination.

Note: Throughout the protocol we have indicated the liquid handling automation in use in the UCSF Center for Advanced Technology, however these steps could be performed on alternative liquid handlers or manually. If you need to adjust reaction volumes, they should scale but verify the changes on a small set of samples first.

If you know the Ct of your samples, you may want to adjust the volume you pool for each sample to account for the trend that higher Ct samples typically require more reads to achieve similar coverage/depth as lower Ct samples.

MATERIALS TEXT

LunaScript RT SuperMix Kit **New England Biolabs Catalog #E3010L** Step 3

NEBNext® Ultra™ II Q5 Master Mix **New England Biolabs Catalog #M0544L** Steps 7 and 11

Q5 Hot Start High-Fidelity 2X Master Mix - 500 rxns **New England Biolabs Catalog #M0494L** Step 7

Agencourt AMPure XP **Beckman Coulter Catalog #A63880** Step 17

Primer sequences - IDT oPool order form

COVID_ARTIC_tailed_TruSeq_275amplicon.xlsx

cDNA generation 45m

1 **Important!** This step must be performed in a RNase free, pre-PCR environment in which post PCR COVID-19 amplicons are not present, to minimise risk of sample contamination.

Decontaminate bench surfaces, pipettes and gloves with RNase ZAP before starting work. Keep reagents and samples chilled throughout the process.

2 Defrost at least 10ul RNA. Store at 4C

3 LunaScript RT SuperMix Kit **New England Biolabs Catalog #E3010L**

Add 8ul RNA to 2ul and mix by pipetting.

4 Seal and briefly centrifuge plate.

5 Place plate on a thermocycler and run the following program:

A	B
Temperature	**Time**
25°C	2 minutes
55°C	20 minutes
95°C	1 minute
4°C	∞
Lid temp: Tracking	

If using a Labcyte Echo, we perform the RT reaction in an LDV source plate with a flat block thermocycler and transfer cDNA directly into the ARTIC multiplex PCR reactions in the next step.

275bp ARTIC cDNA amplification 4h

6 **Note:** Primers for this protocol were modified from https://www.protocols.io/view/covid-19-artic-v3-illumina-library-construction-an-bh4zj8x6. Note that amplicons 109 and 100 have a shared primer region that results in a 15bp overlap that will be unrecoverable. Partial TruSeq R1 or R2 were added to the forward and reverse primers in the pool. Primer sequences and additional information can be found here: **COVID_ARTIC_tailed_TruSeq_275amplicon.xlsx**
These primers can be ordered individually or as an IDT oPool using the the linked form. You can get 50pmol/oligo (one copy per oligo) or 100pmol/oligo (two copies per oligo) if you double each line in the file. These will be enough for 500 or 1000 samples using 10ul PCR reactions. For larger numbers of samples, it will be more cost effective to order each oligo separately.

Create a 10uM pool for primer set 1 and primer set 2.

7 Prepare the two multiplexed SARS-CoV-2 master mixes:

A	B
Multiplexed master mix	**Vol/PCR RXN (µl)**
NEB Q5 Hot Start High-Fidelity mix*	5
Pool 1 or Pool 2 primer mix (10uM)	1.6
Nuclease-free water	2.4
Total	**9**

Aliquot 9ul of Pool 1 and Pool 2 mix into two separate 384-well PCR plates.
*NEB Ultra II Q5 mix may also be used instead, but temperatures may need to be adjusted.

8 Add 1ul of cDNA template to each PCR plate. Seal the plates, vortex to mix, and spin down.

9 **Note:** Amplification should ideally be performed in a different lab to minimize the risk of contaminating other samples.

As noted in the original protocol (https://www.protocols.io/view/covid-19-artic-v3-illumina-library-construction-an-bh4zj8x6), you may need to adjust the annealing/extension temperature on your own thermocycler.

A	B	C
Step	**Temperature**	**Time**
1	98°C	30 seconds
2	98°C	15 seconds
3	63°C	5 minutes
4	Repeat steps 2 & 3 for a total of 35 cycles	
5	4°C	∞

If desired, you can check your amplified cDNA by electrophoresis. You should expect products around 300 bp. For high Ct samples, we observe more primer dimers.

10 **PAUSE POINT** Amplified cDNA can be stored at 4°C (overnight) or -20°C (up to a week).

Perform indexing PCR 45m

11

Prepare indexing PCR reaction in 384-well format

A	B
Indexing PCR Mix	**Vol/PCR RXN (µl)**
NEB Ultra II Q5 mix	5
5uM TruSeq i7/i5 Indexing primer mix	2
Water	2
Total	**9**

12 Combine the two cDNA PCR reactions from each sample and dilute 1:100 in water.

13 Add 1ul of the diluted cDNA pool to the indexing PCR mix.

14 Seal plate, vortex to mix, spin down.

15 Thermocycle according to the protocol below.

A	B	C
Step	**Temperature**	**Time**
1	98°C	0:30
2	98°C	0:10
3	65°C	1:15
4	Repeat Step 2&3 for 10 cycles total	
5	65°C	5:00
6	4°C	∞

Pool samples and cleanup 30m

16

We pool samples after PCR and perform a single cleanup on the pool. If desired, each sample can be purified separately, quantified, and then pooled.

Pool 0.5ul of each library. We transfer the PCR reactions into an Echo LDV plate and use the Echo to pool. If transferring manually, you can increase the volume per sample, just make sure to scale the Ampure bead cleanup appropriately.

17 Agencourt AMPure XP **Beckman**

Add a 0.8x volume of **Coulter Catalog #A63880** or similar reagent. Mix by pipeting.

Allow the DNA to bind for 5 minutes.

18 Place the tube on a magnet.

After the beads have bound to the side of the tube, remove the supernatant.

19 While keeping the tube on the magnet, add 70% ethanol without disturbing the beads.

Wait 30 seconds and remove the ethanol.

20 Perform a second ethanol wash

While keeping the tube on the magnet, add 70% ethanol without disturbing the beads.

Wait 30 seconds and remove the ethanol.

21 Wait 5 minutes or until beads are dry.

Resuspend the beads in one volume water or elution buffer.

Wait 30 seconds

Place tube on magnet.

After beads have moved to the side of the tube, transfer eluate to a new tube.

QC samples and sequence 30m

22 Quantify your samples using a method of your choice (such as Qubit) and run your samples on an electrophoresis system such as a BioAnalyzer or TapeStation (you may need to dilute your sample).

You should expect to see your library centered around 400bp.

Samples should be run on an appropriate PE150 system depending on how many total reads are required. For lower Ct samples, 100,000 reads is sufficient. For higher Ct samples, 1,000,000 or more reads may be required. We have sequenced 384 samples on a single NovaSeq SP PE150 lane or 768 on a single NovaSeq S1 lane.

For analysis, make sure you trim reads using the 275bp primer panel sequences instead of the standard 400bp ARTIC panel sequences.

If you have many high Ct samples, primer dimers may be more abundant and a second bead cleanup could be required.