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TGen North high throughput SARS-CoV-2 tiled amplicon sequencing

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DISCLAIMER

ABSTRACT

Our goal for this project was to incorporate and operationalize a lean laboratory processing method that decreases costs, time, and complexity and increases throughput and accessibility for SARS-CoV-2 tiled amplicon sequencing.

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KEYWORDS

SARS-CoV-2, Illumina sequencing, COVID-19, COVID sequencing, SARS-CoV-2 sequencing, high throughput sequencing, cost-efficient sequencing, NextGen sequencing, COVID, Next Generation sequencing, ARTIC V4, ARTIC

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GUIDELINES

SARS-CoV-2 positive clinical samples should be handled in a BSL2 laboratory environment. See the CDC's guidelines for more information https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html

MATERIALS TEXT

BSL2 Equipment

- Biological Safety Cabinet (BSC) class II or class III
- Sample vortexer
- Single-channel pipette, p1000
- Integra Assist Plus pipetting robot (optional) with p300 Voyager adjustable tip spacing pipette **
- ***Hand-pipetting alteration: p10 multichannel pipette and p1000 single channel pipette*
- Incubator with thermal beads, pre-warmed to 70C
- Pipettes single channel: P1000

BSL1 equipment

- KingFisher Flex Purification System **
- ***For alternative method of SPRI cleanup by hand, pipetting method: Beckman Coulter SPRIPlate 96R Ring Super Magnet Plate*
- ***For alternative method of SPRI cleanup by hand, 96-prong magnetic bead extractor: V&P Scientific Manual Pin tool VP 407AM-N1*
- QuantStudio Real-Time PCR System
- Microplate centrifuge
- Microtube centrifuge
- Sample vortexer
- Plate mixer (optional)
- 96-well plate thermal cycler that can accommodate full-skirt plates
- Pipettes single channel: p2, p20, p200, p1000
- Pipettes 8 or 12 channel: p20

Commercial reagents/kits

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A	B	C	D
Product name	**Vendor**	**Catalog number**	**Alternatives okay?**
Proteinase K	American Bio	AB00925-01000	QIAGEN Proteinase K, Cat: 19131
Absolute Ethanol	VWR	EM8.18760.9025	Alternatives not validated
SPRI beads	Beckman Coulter	A63882	Alternatives not validated
DNase I Reaction Buffer	New England Biolabs	B0303S	Alternatives not validated
DNase I (RNase-free)	New England Biolabs	M0303L	Alternatives not validated
Polyethylene glycol 8,000 50% (w/v)	VWR	101443-878	Alternatives not validated
Sodium chloride 5 M in aqueous solution, sterile filtered	VWR	10128-484	Alternatives not validated
Molecular Grade Water	VWR	45001-044	Alternatives not validated
LunaScript	NEB	E3010L	Alternatives not validated
Q5 HF Hot Start	NEB	M0492L	Alternatives not recommended
ARTIC V4 panel	Integrated DNA Technologies		Alternatives not validated
PlexWell 384	SeqWell	PW384	Alternatives not validated
Kapa HiFi HotStart	Roche	KK2602	Alternatives not recommended
illumina - ROX low qpcr mastermix, SYBR Fast, Complete Kit w/ standards	Roche	07960336001	Alternatives not recommended
NextSeq Mid Output 300 cycle	Illumina	20024905	Alternatives not validated

Plastics/Consumables

A	B	C
Product name	**Vendor**	**Catalog Number**
20uL pipette tip refills	Rainin	30389274
200uL pipette tip refills	Rainin	30389276
1000uL pipette tip refills	Rainin	30389272
Mid-skirt PCR plate 96 wells	Labcon	3973-520-000-9
2.0mL microcentrifuge tube	Neptune	3765.S.X
1.7mL microcentrifuge tube	Axygen	MCR-175-C-S
2.0mL microcentrifuge tube O-ring	VWR	525-1159
0.2mL tubes	VWR	732-3485
Reagent Reserviors	USA Scientific	1346-1010
384-well plates	Applied Biosystems	4309849
Thermal cycler seal	Bio-Rad	MSB1001
Optical seal	Applied Biosystems	4311971
Temporary plate seal	Costar	6524

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DISCLAIMER:

Abbreviations

1 **General**

- PPE - Personal Protective Equipment
- BSC - Biological Safety Cabinet
- cDNA - complementary DNA
- RT - reverse transcriptase
- PCR - Polymerase Chain Reaction
- rtPCR - realtime PCR
- RT-PCR - Reverse Transcriptase PCR
- rt RT-PCR - realtime Reverse Polymerase PCR
- Ct - Cycle threshold

Controls

- NEC - Negative Extraction Control
- PEC - Positive Extraction Control

Control Generation

2 **NEC (Negative Extraction Control)**

- Negative media (sterile saline or viral transport media)

PEC (Positive Extraction Control)

- Known concentration of SARS-CoV-2 RNA in a clinical matrix
- Concentration: at or near lower limit of viral input (Ct ~32)
- Clinical matrix: remnant clinical specimens known to be negative for SARS-CoV-2
- Consider using a strain that is not common in the populations being sequenced (i.e. a basal strain)

Sample selection

3 **Verified specimen types**

Known SARS-CoV-2 positive clinical specimens, ≥50uL volume (ideally ≥500uL)

- NP swabs in sterile saline or viral transport media
- Anterior nasal swabs in sterile saline or vital transport media
- Saliva
- Remnant swabs from rapid antigen test platforms, suspended in sterile saline or viral transport media (such as Abbott ID NOW)

**High viral load specimens (≤ Ct 30 on most PCR tests) will yield higher quality data in sequencing workflows. Lower viral load samples may be sequenceable, but often yield less consistent/lower quality results. Additional validation may be necessary to determine a more precise cut-off.

Initial sample processing

4 **Specimen storage recommendations**

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Upon receipt of specimens:

- Refrigerated specimens should be maintained at 2-8°C if they will be processed within 7 days. Otherwise, they should be frozen at -20°C for longer term storage.
- Specimens that are received frozen should be kept frozen at -20°C until ready to process. They may be thawed at 4C or at room temperature
- Very long term storage of specimens (storage upon completion of processing) should occur at -70°C

Metadata to record (for research applications, PHI should NOT be recorded)

- County of sample collection
- Date of collection

5 Transfer to long term storage vessel

If necessary, transfer sample volume into a smaller, long term storage container (i.e. sterile 2mL screw-cap tube with O-ring)

Use a BSC and appropriate PPE while handling active agent

- Both the original specimen and a new 2mL screw-cap tube should be given corresponding labels prior to volume transfer
- Use a p1000 pipette to transfer up to 1,500uL from the original specimen container to a 2mL screw-cap tube

Sample Array and inactivation

6 Sample array and inactivation

If applicable, separate specimens into high and low volume processing sets

- ≥50uL = Low volume workflow
- ≥500uL = High volume workflow

Proceed to appropriate inactivation method below

Step 6 includes a Step case.

High (≥500uL)

Low ≥50uL

Sample Purification + DNase treatment

step case

High (≥500uL)

In a KingFisher 96 deep-well plate, fill appropriate wells with 10uL proteinase K

**May be performed by Integra Assist Plus repeat dispense program

- wells filled with proteinase K should correspond with the number of low volume samples to be processed, and should include two extra wells for a PEC and an NEC (i.e. 96 wells filled with 6.5uL proteinase K would correspond to 94 sample wells, plus one NEC well and one PEC well)
- can be prepared up to 72 hours in advance advance if the plate is well-sealed to prevent evaporation (store at 4°C). Briefly centrifuge plate at ≥1,000rcf to collect contents at bottom of wells.

Array 500uL sample into each well of the plate

**May be performed by Integra Assist Plus transfer program (300uL pipette attachment requires 2x 250uL transfers)

- Add 500uL PEC to a well containing proteinase K to a well containing proteinase K
- Add 500uL NEC to a well containing proteinase K
- Add 50uL NEC to a well containing proteinase K

Seal with thermal cycler compatible sealing tape

Mix

- Place plate on a plate mixer, ≥850 rpm for ≥1 min
- Alternatively, mix by pipetting 2x after each 500uL sample addition (no plate centrifugation required)

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Briefly centrifuge plate at ≥1,000rcf to collect contents at bottom of wells.

Heat inactivate in incubator (ensure thermal beads have reached a temperature of 70°C, not just the incubator itself)
70°C for 60 mins

- Sample temperature must reach 56°C for 30 minutes for total inactivation of virus.

7 DNase treatment is a required part of the workflow, as it removes significant amounts of background human DNA from your viral samples.

REAGENT PREPARATION

DNase reagent mixture
**keep refrigerated when not in use, use within 24 hours
Per plate (96 samples, ~10% extra)

- 525uL 5uL DNase I Enzyme
- 525uL DNase I Reaction Buffer
- 4.2mL Molecular Grade Water

20% PEG, 2.5M NaCl solution
**Store at room temperature, use within 1 month
**You will use ~5mL per plate of 96 samples

- 200mL 50% PEG 8000
- 250mL 5M NaCl
- 8.5mL 1X Tris EDTA
- 5Bring total volume to 500mL with Molecular Grade Water

70% EtOH
**store at room temperature, use within 1 week
**you will use ~45mL per plate of 96 samples

- 35mL 100% Ethanol
- 15mL Molecular Grade Water

Proceed to appropriate protocol below

500uLKingFisher-protocol.pdf
50uLKingFisher-protocol.pdf

Step 7 includes a Step case.
KingFisher500uL
KingFisher 50uL
By hand, 50uL

Sample qualification

step case

KingFisher500uL

PREPARE REAGENT PLATES

EtOH wash 1,2,3 - Standard Plate

- 150uL 70% EtOH per well

DNase - Standard plate

- 50uL DNase reagent mixture per well

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Elution

- 50uL molecular grade water per well

Sample plate

- Add 60uL SPRI beads (mixed vigorously) to deep-well plate containing proteinase K lysate
- Add 300uL 100% EtOH to deep-well plate containing proteinase K lysate

PROTOCOL

linked above, 500uLKingFisher-protocol.pdf

8 **Establishing an early quality measure**

It is advantageous to define a pre-library prep quality measure that is predictive of successful sequencing. Viral load is recommended. TGen has determined that a Ct value of ≤34 on a SARS-CoV-2 N gene target RT-PCR assay is predictive of a high quality genome. However, validation may yield a different cutoff in a different lab. A predictive cutoff can be established by plotting pre-library prep viral load quantities against their resulting genome quality measures. A drop-off in genome quality should be apparent at some point where viral load is lower.

Using cutoffs efficiently

If a large percentage (>20%) of samples are expected to be low viral load and thus not expected to yield quality data, it is advisable to remove low viral load samples from the workflow at this point. This will prevent wasted reagent and labor that would occur from proceeding with these poor quality samples.

If ≥80% of samples are expected to yield high quality data, the labor investment in removing the poor quality samples surpasses the reagent/labor waste incurred by processing them. For this reason, it is advised not to remove individual samples from the larger set.

cDNA synthesis

9 **NEB LunaScript RT SuperMix Kit**

Combine the following in a 96-well thermal cycler compatible microplate
**The following is a validated partial reaction (50% decrease from NEB recommended volumes)

- 2uL LunaScript RT SuperMix 5X
  - 96-well plates containing 2uL SuperMix per well may be prepared ahead of time, sealed, and stored at -20C
- 8uL RNA (post-DNase treatment/purification product)

Mix

- Plate vortexer ≥850rpm for ≥30 seconds
- Alternatively, pipette mix 3-4 times with every addition of 8uL RNA

Centrifuge briefly

Thermal cycler program:

Heated Lid:105C
Volume: 10uL

CYCLE STEP	TEMPERATURE	TIME	CYCLES
1. Primer Annealing	25°C	2 minutes	1
2. cDNA Synthesis	55°C	10 minutes	1
3. Heat Inactivation	95°C	1 minute	1
4. Hold	10°C	∞	

ARTIC V4 PCR

10 **ARTIC V4 Primer Panel Info**

Primer Design and pooling concentrations available on GitHub https://github.com/artic-network/artic-ncov2019/tree/master/primer_schemes/nCoV-2019/V4

- Primer sequences: https://github.com/artic-network/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V4/SARS-CoV-2.primer.bed

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- Pooling instructions: https://github.com/artic-network/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V4/README

20x mix instructions:
- Pool 100uM stocks of primers according to GitHub README instructions
- Dilute pools by a factor of 2.62158108 (1 part primers, 2.62 parts 1x Tris EDTA)

Resulting concentrations in 20x primer mix
- 17, 27, 30, 61, 74 (5x volume) = 1500nM
- 13, 45, 79 (2x volume) = 600nM
- Everything else (1x volume) = 300nM

11 Tiled amplicon PCR instructions

Prepare sufficient volume of mastermix for the number of samples being processed, plus 10% extra for pipette error. This mastermix will need to be created twice, once for primer pool 1 and again for primer pool 2.

MasterMix calculations, per sample
- Molecular Grade Water 8.75 uL
- Primer Mix (20x) 1.25 uL
- Q5 12.5 uL

MasterMix calculations, plate of 96 plus 10%
- Molecular Grade Water 924 uL
- Primer Mix (20x) 132 uL
- Q5 1320 uL

11.1 PCR Assay Assembly

Combine
- 22.5uL Master Mix
- 2.5uL cDNA

Mix
- Plate vortexer ≥850rpm for ≥30 seconds
- Alternatively, pipette mix 3-4 times with every addition of 8uL RNA

Centrifuge Briefly

Thermal cycler program:
Heated Lid:105C
Volume: 25uL

CYCLE STEP	TEMPERATURE	TIME	CYCLES
1. Heat Activation	98°C	30 sec	1
2. Denaturation	98°C	15 sec	
3. Annealing	65°C	5 mins.	Repeat steps 2-3 for a total of 35 cycles
4. Hold	10°C	∞	

PlexWell 384 protocol

12 Pooling PCR product
- For each sample, combine 25uL ARTIC pool 1 product with 25uL ARTIC pool 2 product (entire volumes).
- Perform a 1:9 dilution on the pool 1/pool 2 combination (i.e. 5uL pooled product, 45uL molecular grade water)

12.1 Proceed to PlexWell protocol:

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- perform as written

plexWell_384_and_96_Library_Preparation_Kit_User_Guide_v20210609.pdf

Load on Illumina Sequencer

13 Sequencing coverage recommendations

TGen routinely uses a NextSeq Mid Output 300 cycle kit, with up to 576 samples per run. This gives ~65 megabases of sequencing data to each sample.

- More samples per run may be independently validated by gradually increasing cluster density. Run quality metrics should be closely monitored, and increases in cluster density should be halted when metrics show a marked decline or go out of spec.
- As long as ≥65Mb per sample is achieved, scaling to smaller or larger sequencing kits is possible.

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