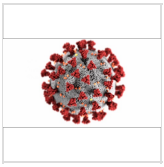

Version 1

Apr 29, 2020

SARS-CoV-2 Sequencing on Illumina MiSeq Using ARTIC Protocol: Part 1 - Tiling PCR V.1

Joel Sevinsky[1], Arian Nassiri[2], Erin Young[3], Heather Blankenship[4], Kevin Libuit[2], Kelly Oakeson[3], Lauren Turner[2], StaPH-B Consortium[5]

[1]Theiagen Consulting LLC, [2]Virginia Division of Consolidated Laboratory Services, [3]Utah Public Health Laboratory, [4]Michigan Department of Health and Human Services, [5]State Public Health Bioinformaticians

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Coronavirus Method Development Community StaPH-B view 1 more group

Joel Sevinsky
Theiagen Consulting LLC

ABSTRACT

This protocol is an adaption of several circulating protocols on SARS-CoV-2 sequencing using the ARTIC protocol. Its purpose is to simplify things for the average state public health laboratory, using equipment and expertise they currently posess, most likely from their funded PulseNet activities.

This protocol is derived from other works, including:

https://www.protocols.io/view/ncov-2019-sequencing-protocol-bbmuik6w
https://www.protocols.io/view/ncov-2019-sequencing-protocol-v2-bdp7i5rn

pcr-tiling-ncov-PTC_9096_v109_revE_06Feb2020-minion.pdf

STEPS MATERIALS

NAME	CATALOG #	VENDOR
DNA LoBind 1.5mL microcentrifuge tubes	13-698-791	Fisher Scientific
AMPure XP	A63881	Beckman Coulter
Random primer mix	S1330S	New England Biolabs
Deoxynucleotide Solution Mix - 8 umol of each	N0447S	New England Biolabs
SuperScript™ IV Reverse Transcriptase	18090010	Thermo Fisher
96 well LoBind PCR plates Semi-skirted	0030129504	Eppendorf
RNaseOUT™ Recombinant Ribonuclease Inhibitor	10777019	Thermo Fisher Scientific
Q5 Hot Start High-Fidelity 2X Master Mix - 100 rxns	M0494S	New England Biolabs
RT-PCR Grade Water	AM9935	Thermo Fisher
Qubit dsDNA HS Assay Kit	Q32854	Thermo Fisher Scientific

EQUIPMENT

NAME	CATALOG #	VENDOR
Qubit Fluorometer	Q33238	Thermo Scientific

ARTIC Protocol - Prepping Nucleic Acid

1 Getting RNA ready for cDNA Creation

In this section we cover the process of preparing your nucleic acid/RNA extractions, from your qPCR diagnostic test, to be used in viral sequencing. You will need to have Ct values for each of your specimens because this will determine your dilution factor prior to starting your cDNA preparation. Dilute your sample based on the chart below:

Citation: Joel Sevinsky, Arian Nassiri, Erin Young, Heather Blankenship, Kevin Libuit, Kelly Oakeson, Lauren Turner, StaPH-B Consortium (04/29/2020). SARS-CoV-2 Sequencing on Illumina MiSeq Using ARTIC Protocol: Part 1 - Tiling PCR. https://dx.doi.org/10.17504/protocols.io.bfefjjbn

qPCR Ct**	Dilution Factor
18-35	none
15-18	1:10
12-15	1:100

Dilution factor guide

You can use the attached worksheet to help with sample organization: Initial Sample Dilution Sheet.pdf

Initial Sample Dilution Sheet.xlsx

****NOTE: If you do not have Ct values from your diagnostic test, you can use the RNA extractions without dilution. An alternative approach that has been successful that helps with througput is to use every samples undiluted, and only if it fails to sequence correctly go back and dilute. Some labs have been reporting that greater than 90% of their specimens will sequence fine undiluted.**

ARTIC Protocol - cDNA Preparation - Reverse Transcription

2 cDNA/Reverse Transcription Section

Date/Initials:_____

In this section we cover the process of taking your nucleic acid extraction from your qPCR diagnostic test and use it as starting material for the sequencing.

2.1 [ ] In a PCR hood, mix the following reagents and add to a **0.2 ml** PCR tube on a cold block plus **11 µl** of RNA sample:

Reagent	Volume (uL)	MM for N+2 samples
60 uM random hexamers and anchored polyT(23)	1.0	
10mM dNTPs	1.0	
Total	**2.0**	

Master mix calculations

Each reaction should have **13 µl** when mixed. **If using master mix, it is recommended to add the 2 µl of the master mix to the PCR tube first, then add the 11 µl of RNA to help prevent contamination.**

Random primer mix
by New England Biolabs
Catalog #: S1330S

Lot# _____ Exp. Date _____

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Deoxynucleotide Solution Mix - 8 umol of each
by New England Biolabs
Catalog #: N0447S

Lot# _____ Exp. Date _____

2.2 [ ] Mix gently, spin down, and return to **On ice** .

2.3 [ ] Preheat Thermocycler to **65 °C** , with heated lid at **105 °C**

2.4 [ ] Incubate the reaction at **65 °C** for **00:05:00** , followed by an immediate snap-cool on **On ice** for at least **00:01:00** .

2.5 [ ] In a clean **1.5 ml** LoBind tube (96 well plates can also be used) on **On ice** , mix together the following reagents:

Reagent	Volume (uL)	MM for N+2 samples
SuperScript IV RT 5X Buffer**	4.0	
100mM DTT**	1.0	
RNaseOUT RNase inhibitor	1.0	
Superscript IV Reverse Transcriptase**	1.0	
Total	**7.0**	

Master mix for RT reaction.

****Note: All these reagents are part of the SuperScript IV Reverse Transcriptase kit.**

RNaseOUT™ Recombinant Ribonuclease Inhibitor
by Thermo Fisher Scientific
Catalog #: 10777019

Lot# _____ Exp. Date _____

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SuperScript™ IV Reverse Transcriptase
by Thermo Fisher
Catalog #: 18090010

Lot# _____ Exp. Date _____

DNA LoBind 1.5mL microcentrifuge tubes
by Fisher Scientific
Catalog #: 13-698-791

96 well LoBind PCR plates Semi-skirted
by Eppendorf
Catalog #: 0030129504

2.6 [ ] After the RNA sample has cooled for at least **00:01:00** , longer if needed to make master mix, add **7 µl** of the above master mix to the sample.

2.7 [ ] Mix gently by flicking, and spin down. Return tube to **On ice** .

2.8 [ ] Preheat thermocycler to **42 °C** , with heated lid at **105 °C**

2.9 [ ] Incubate sample using the following COVID WGS Reverse Transcription program:

Step	Temp	Time	Cycle
Reverse Transcription	42 C	50:00	1
RT Inactivation	70 C	10:00	1
Cool	4 C	Hold	Hold

SARS-CoV-2 Reverse Transcription Program

ARTIC Protocol - Tiled PCR Section

3 Tiled PCR Section Date/Initials:_____

This section outlines the process for the tiled PCR approach from the ARTIC protocol. A seperate document will be provided outlining how to order primers and make the two different primer pools needed for this section. Hopefully most first time labs will receive aliquots of both Pool A and Pool B from labs that have successfully completed this protocol before to help with any potential troubleshooting.

Citation: Joel Sevinsky, Arian Nassiri, Erin Young, Heather Blankenship, Kevin Libuit, Kelly Oakeson, Lauren Turner, StaPH-B Consortium (04/29/2020). SARS-CoV-2 Sequencing on Illumina MiSeq Using ARTIC Protocol: Part 1 - Tiling PCR. https://dx.doi.org/10.17504/protocols.io.bfefjjbn

3.1 [ ] Set up two individual reactions using primer pool A and primer pool B in **0.2 ml** PCR tubes according to the following table:

Reagent	Pool A (uL)	MM for N+2 samples	Pool B (uL)	MM for N+2 samples
Q5 Hot Start HiFi 2x MM	12.5		12.5	
Primer pool at 10uM (A or B)**	3.7		3.7	
Nuclease-free water	6.3		6.3	
Total	**22.5**		**22.5**	

Master Mix for Tiled PCR

**See protocol on primer design for SARS-CoV-2. If this is your first attempt, it would be best to receive aliquots of the primers from a lab that has successfully sequenced SARS-CoV-2 first.

Q5 Hot Start High-Fidelity 2X Master Mix - 100 rxns
by New England Biolabs
Catalog #: M0494S

Lot# _____ Exp. Date _____

RT-PCR Grade Water
by Thermo Fisher
Catalog #: AM9935

Lot# _____ Exp. Date _____

Any PCR grade water will do in this step. Not necessary to use the reagent listed.

3.2 [ ] Add **2.5 µl** sample cDNA to each pool.

3.3 [ ] Mix gently and spin down prior to loading on the thermocycler.

3.4 [ ] Run the following thermocycler program:

Step	Temp	Time	Cycles
Initial Denaturation	98°C	0:30	1

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Denaturation	98°C	0:15	25 or 35**
Anneal and Extension	65°C	5:00	25 or 35**
Cool	4°C	Hold	Hold

SARS-CoV-2 Tiled PCR - see note below.

****Note: If starting RNA samples had a qPCR Ct value in the range of 12-21, use 25 cycles.**
If starting RNA samples had a qPCR Ct values in the range of 21-35, use 35 cycles. An alternative approach that has been successful that helps with througput is to run every specimen undiluted and for 35 cycles, and only if it fails to sequence correctly go back and dilute and/or run for fewer cycles. Some labs have been reporting that greater than 90% of their specimens will sequence fine undiluted for 35 cycles.

ARTIC Protocol - Clean-Up and Size Selection

4 Section for Clean-Up and Size Selection

Date/Initials:_____

This process is similar to the bead clean-ups performed for the PulseNet WGS protocol. The same beads and magnets may be used, although it is recommended to have seperate beads to help prevent contamination.

4.1 [ ] Combine the **25 µl** reaction from Pool A and the **25 µl** reaction from Pool B into a new **1.5 ml** LoBind tube. One tube per sample.

DNA LoBind 1.5mL microcentrifuge tubes
by Fisher Scientific
Catalog #: 13-698-791

4.2 [ ] Re-suspend AMPure XP beads by vortexing.

AMPure XP
by Beckman Coulter
Catalog #: A63881

Lot# _____ Exp. Date _____

The AMPure XP is available in **5 ml** , **60 ml** , and **450 ml** sizes. Please choose the appropriate size for your throughput.

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4.3 [ ] Add **50 µl** of re-suspended AMPure XP beads to the reaction and mix.

4.4 [ ] Incubate on a rotator mixer for **00:10:00** at **Room temperature** .

4.5 [ ] Prepare **500 µl** **80 % volume** ethanol using the following calculation:

Sample# + 1: _____

0.5ml x Sample# = _____ mL total volume

mL total volume x 0.8 = _____ mL EtOH

Total volume _____mL - _____mL EtOH = _____mL H2O

4.6 [ ] Spin down sample and pellet the beads on a magnet** for approximately **00:05:00** . Keep tubes on the magnet** and pipette off supernatant.

****Use the magnetic stands from your PulseNet protocols.**

4.7 [ ] While on the magnet, wash beads with **200 µl** freshly prepared **80 % volume** EtOH without disturbing the pellet. Rotate the tube to allow the bead pellet to migrate towards the opposite side of the tube. Remove EtOH

4.8 [ ] Repeat previous step.

4.9 [ ] Spin down and place the tubes back on the magnet. Pipette off any residual ethanol and allow to dry for approximately **00:00:30** . Take care to not over dry the pellet.

4.10 [ ] Remove tubes from the magnet and re-suspend pellet in **30 µl** of nuclease-free water**.

****Use whatever nuclease free water was used in previous steps.**

4.11 [ ] Incubate at **Room temperature** for approximately **00:02:00** .

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4.12 [ ] Pellet the beads on a magnet until eluate is clear and colorless

4.13 [ ] Remove ~ **30 µl** of eluate and place in a clean **1.5 ml** LoBind tube.

4.14 [ ] Quantify eluted sample on Qubit fluorometer or similar instrument and store completed PCR amplified cDNA prep at **-20 °C** .

Qubit Fluorometer
Fluorometer
Invitrogen Q33238

Qubit dsDNA HS Assay Kit
by Thermo Fisher Scientific
Catalog #: Q32854

Earlier versions of the Qubit, or any other method for accurate DNA quantitation can be used. Most labs should have this equipment available in their NGS sections.

4.15 [ ] PCR amplified cDNA is now ready for Illumina library preparation. Please proceed to Part 2 for DNA Flex Library Preparation.

NOTE: For those of you familiar with the PulseNet protocols for WGS of bacterial pathogens, you are at the equivalent stage where you have extracted you DNA from your colony and are ready to begin library preparation, usually with DNA Flex.

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