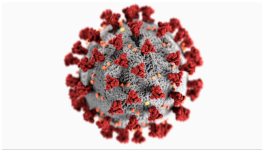
Apr 29, 2020 Version 1

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



Virus Evolution

In 2 collections

DOI
https://dx.doi.org/10.17504/protocols.io.bfefjjbn

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 Genomics; [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

Coronavirus Method De... StaPH-B 2 more workspaces

Joel Sevinsky
Theiagen Genomics

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.bfefjjbn

External link: https://doi.org/10.1093/ve/veac104

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

Manuscript citation:
Halfmann PJ, Minor NR, III LAH, Maddox R, Moreno GK, Braun KM, Baker DA, Riemersa KK, Prasad A, Alman KJ, Lambert MC, Florek K, Bateman A, Westergaard R, Safdar N, Andes DR, Kawaoka Y, Fida M, Yao JD, Friedrich TC, O'Connor DH (2022) Evolution of a globally unique SARS-CoV-2 Spike E484T monoclonal antibody escape mutation in a persistently infected, immunocompromised individual. Virus Evolution 9(2). doi: 10.1093/ve/veac104

Protocol status: In development
We are still developing and optimizing this protocol. Comments and feedback appreciated.

Created: April 21, 2020

Last Modified: April 29, 2020

Protocol Integer ID: 36007

Keywords: sequencing protocol, pcr this protocol, using artic protocol, protocols on sar, artic protocol, circulating protocol, tiling pcr, pcr, protocol, pulsenet activity, ncov, sequencing

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_rev...

Materials

STEP MATERIALS

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

Protocol materials

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

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:

	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

****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 **New England Biolabs Catalog #**S1330S
Lot# _____ Exp. Date _____

Deoxynucleotide Solution Mix - 8 umol of each **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

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

RNaseOUT™ Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific Catalog #**10777019

Lot# _____ Exp. Date _____

SuperScript™ IV Reverse Transcriptase **Thermo Fisher Catalog #**18090010

Lot# _____ Exp. Date _____

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

96 well LoBind PCR plates Semi-skirted **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.

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

Note

**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 **New England Biolabs Catalog #**M0494S

Lot# _____ Exp. Date _____

RT-PCR Grade Water **Thermo Fisher Catalog #**AM9935

Lot# _____ Exp. Date _____

Note

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
	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

**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 **Fisher Scientific Catalog #**13-698-791

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

AMPure XP **Beckman Coulter Catalog #**A63881
Lot# _____ Exp. Date _____

Note

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

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 [M] 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.

Note

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

4.7 [ ] While on the magnet, wash beads with 200 µL freshly prepared [M] 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**.

Note

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

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

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 .

Equipment

Qubit Fluorometer	NAME
Fluorometer	TYPE
Invitrogen	BRAND
Q33238	SKU
https://www.thermofisher.com/order/catalog/product/Q33238#/Q33238	LINK

Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32854

Note

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

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.