VIRUSHUNTER™ ARTIC SARS-CoV-2 Whole Genome Sequencing Assay

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DISCLAIMER

ABSTRACT

The VirusHunter ARTIC SARS-CoV-2 Whole Genome Sequencing Assay uses a modified version of the sample prep method developed by Josh Quick and the ARTIC Network using the same multiplex RT-PCR primers (https://www.protocols.io/view/ncov-2019-sequencing-protocol-v3-locost-bh42j8ye). The protocol begins with RT-PCR using two different primer pools for each sample. The pools are then combined and the RT-PCR products are fragmented using DNase I to generate small fragments that can more efficiently hybridize to QuadCore DNA chips from Centrillion Technologies. Following hybridization, chips are stained, scanned with the Summit scanner, and analyzed using VirusHunter software. Analysis workflows are not described herein (check out our GitHub page for analysis software).

Initial results of v1 of this protocol have been reported here:
https://pubs.acs.org/doi/abs/10.1021/acs.langmuir.0c02927

with improvements to the calling algorirthm reported here:
https://www.biorxiv.org/content/10.1101/2021.05.11.443659v1

This is an updated version of the protocol with improved coverage and accuracy. Expected coverage is >99% with >99.9% possible for high-quality samples. Expected accuracy is >99.9% with >99.99% possible for high-quality samples.

ATTACHMENTS

Highly Accurate Chip-Based Resequencing of SARS-CoV-2 Clinical.pdf

DOI

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EXTERNAL LINK

https://www.centrilliontech.com/

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PROTOCOL CITATION

Kendall Hoff 2021. VIRUSHUNTER™ ARTIC SARS-CoV-2 Whole Genome Sequencing Assay. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bud4ns8w

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Hoff, Kendall, et al. "Highly Accurate Chip-Based Resequencing of SARS-CoV-2 Clinical Samples." Langmuir (2021).

KEYWORDS

null, COVID-19

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PROTOCOL INTEGER ID

49308

GUIDELINES

The assay has not been evaluated by the FDA.

The VirusHunter™ ARTIC-SARS-CoV-2 Assay is intended for use only with previously positive patient samples. Samples with Ct values below 25 are considered ideal, however, samples with Ct values between 25-30 are generally considered acceptable. This assay has been evaluated for samples with Ct values up to 32 with excellent coverage and accuracy.

The VirusHunter™ ARTIC-SARS-CoV-2 workflow should be performed by qualified and trained staff to avoid the risk of erroneous results. Use separate areas for the preparation of patient samples and controls to prevent false positive results. Samples and reagents must be handled under a laminar airflow hood or biological safety cabinet.

Follow standard precautions. All patient specimens and positive controls should be considered potentially infectious and handled accordingly.

Use personal protective equipment (PPE) consistent with current guidelines for the handling of potentially infectious samples.

Perform all manipulations of live virus samples within a Class II (or higher) biological safety cabinet (BSC).

Do not eat, drink, smoke, apply cosmetics or handle contact lenses in areas where reagents and human specimens are handled.

Always use pipette tips with aerosol barriers. Tips that are used must be sterile and free from DNases and RNases.

Refer to Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with 2019-nCoV https://www.cdc.gov/coronavirus/2019-nCoV/lab-biosafety-guidelines.html.

Modifications to assay reagents, assay protocol, or instrumentation are not permitted once an established protocol has been validated within a laboratory.

Reagents must be stored and handled as specified.

Do not use reagents after the indicated expiry date.

Dispose of waste in compliance with local, state, and federal regulations.

Amplification technologies such as PCR are sensitive to the accidental introduction of PCR product from previous amplification reactions. Incorrect results could occur if either the clinical specimen or the real-time reagents used in the amplification step become contaminated by the accidental introduction of amplification product (amplicon). Workflow in the laboratory should proceed in a unidirectional manner.

Maintain separate areas for assay setup and handling of nucleic acids.

Always check the expiration date prior to use. Do not use expired reagents. Do not substitute or mix reagents from different kit lots or from other manufacturers.

Change aerosol barrier pipette tips between all manual liquid transfers.

During the preparation of samples, compliance with good laboratory techniques is essential to minimize the risk of cross-contamination between samples, and the inadvertent introduction of nucleases into samples during and after the extraction procedure. Proper aseptic technique should always be used when working with nucleic acids.

Maintain separate, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips) for assay setup and handling of extracted nucleic acids.

Wear a clean lab coat and powder-free disposable gloves (not previously worn) when setting up assays.

Change gloves between samples and whenever contamination is suspected.

Keep reagent and reaction tubes capped or covered as much as possible.

Primers, probes (including aliquots), and enzyme master mix must be thawed and maintained on cold block at all times during preparation and use.

Work surfaces, pipettes, and centrifuges should be cleaned and decontaminated with cleaning products such as 10% bleach, “DNAZap™” or “RNase AWAY®” to minimize risk of nucleic acid contamination. Residual bleach should be removed using 70% ethanol.

RNA should be maintained on cold block or on ice during preparation and use to ensure stability.

Dispose of unused kit reagents and human specimens according to local, state, and federal regulations.

MATERIALS TEXT

Chips and Trays

Reagent Label	Description	Quantity Provided	Storage and notes
Wash tray	Disposable tray with 20mL wash chamber	3	Room temperature (20-25°C)
96-well tray	Disposable 96-well tray with 50-60µL well capacity	3	Room temperature (20-25°C)
Scan tray	Glass-bottom tray for imaging microarrays	1	Room temperature (20-25°C)
96-well VirusHunter™ SARS-CoV-2 chip plate	96 microarray plate with 96-well hybridization tray	1	Store refrigerated in a dehumidified environment

Molecular Reagents

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Required Reagent	Recommended Product Name	Vendor	Catalog #
RNA Extraction Kit	MagMAX Viral and Pathogen Nucleic Acid Isolation Kits	Thermo Fisher	A48383, A48310, A42352
Total RNA Control (Human)	Total RNA Control (Human)	Thermo Fisher	4307281
Inactivated Virus Control	ZeptoMetrix SARS-CoV-2 Isolate USA-WA1/2020	Zeptometrix	NATSARS(COV2)-ST
LunaScript™ RT SuperMix Kit	LunaScript™ RT SuperMix Kit	NEB	E3010
Q5® Hot Start High-Fidelity DNA Polymerase	Q5® Hot Start High-Fidelity DNA Polymerase	NEB	M0493
DNase I	Deoxyribonuclease I from bovine pancreas	Sigma	D4236
1M Tris-HCl pH 7.5	UltraPure™ 1 M Tris-HCl Buffer, pH 7.5	Thermo Fisher	15567027
2M Magnesium Chloride	Magnesium chloride solution (BioUltra)	Sigma	68475-100ML-F
1M Calcium Chloride	Calcium chloride solution (BioUltra)	Sigma	21115-250ML
0.5M EDTA	EDTA (0.5 M), pH 8.0, RNase-free	Thermo Fisher	AM9261, AM9262
Biotin-11-dUTP	Biotin-11-dUTP	Jena Biosciences	NU-803-BIOX-S, NU-803-BIOX-L
dNTPs (25mM each)	dNTP (25 mM each) ≥99% HPLC	Genscript	C01581
Cy3-Labeled Streptavidin	Streptavidin - Cy3	Thermo Fisher	434315
Saline-Sodium Citrate Buffer	SSC (20X), RNase-free	Thermo Fisher	AM9763, AM9770, AM9765
HEPES Buffer pH 8.0	HEPES, 0.5M buffer soln., pH 8.0	Alfa Aesar	J63002-AE
20% Ficoll 400	Ficoll® solution, Type 400, 20% in H2O	Millipore Sigma	F5415-25ML, F5415-50ML
TWEEN20	TWEEN® 20	Millipore Sigma	P9416-50ML, P9416-100ML
Nuclease-free Water	Nuclease-free Water	IDT	11-05-01-04
10x PBS	PBS (10X), pH 7.4	Thermo Fisher	70011044
20mg/mL BSA	Bovine Serum Albumin solution	Sigma	B8667
Biotinylated Anti-Streptavidin Antibody	Anti-Streptavidin Antibody, Biotinylated	Vector Labs	BA-0500-.5

Equipment and Disposables

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A	B	C
Item	**Quantity**	**Specifications/Description**
Summit™ Scanner (Centrillion Technologies Inc. P/N CEN-Summit-01)	1	Scanning Fluorescence Confocal Microscope capable of scanning QuadCore™ chips with computer and compatible VirusHunterLab™ software
Hybridization oven	2	Laboratory oven with a size capacity of at least 8x8x4 inches (WxDxH). Set point range of at least +5ºC to 50 ºC with precision of at least 0.5ºC.
Thermal cycler	1	96-well block format for 0.2mL PCR plates and tubes (volume range of 10-100µL) capable of maintaining temperature at 4.0-99.9 ºC with temperature uniformity of +/- 0.5ºC (30 seconds after reaching 95ºC).
Centrifuge, with a rotor for microplates	1	Centrifuge capable of maintaining 1,000 x g with a rotor to fit 96-well PCR plates and QuadCore™ chip plates.
96-well PCR plates	N/A	VWR #83007-374 or equivalent
Tape or foil to seal 96-well plates	N/A	VWR #60741-070 or equivalent
Low binding microcentrifuge tubes	N/A	Eppendorf #60741-070 or quivalent
1L Nuclease-free bottles	N/A	Grenier #952700 or equivalent
50mL Conicals	N/A	Corning #CLS430828-500EA or equivalent
Reagent Reservoirs	N/A	USA Scientific #NC9895604 or equivalent

1 **RNA Extraction: this protocol is for the sequencing of SARS-CoV-2 from previously positive patient samples. Leftover RNA from qPCR testing can be used for this assay assuming proper storage/handling.**

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2 RT

In the PCR cabinet, add **2.2 µl** of the 5xLunaSript RT SuperMix to PCR tubes or plates.

Add **8.8 µl** of your template RNA in a distinct sample addition cabinet. Gently mix and pulse spin.

Transfer your samples to a thermal cycler and incubate as follows:

A	B	C
Step	**Temperature**	**Time**
Annealing	25°C	2 minutes
Reverse Transcription	55°C	60 minutes
Heat Inactivation	95°C	1 minute
Hold	4°C	

3 PCR

In the PCR cabinet, prepare two PCR master mixes (one with Pool 1 primers and one with Pool 2 primers) for each sample. Gently mix and pulse spin.

A	B	C
Reagent	**Volume (µL): total = 25µL**	**Number of Samples +10%**
Nuclease-free water	8.85	
5x Q5 Reaction buffer	5	
Biotin-11-dUTP (1mM)	1.25	
25mM dNTPs	0.4	
Primer Mix 1 or 2 (10µM)	4	
Q5 Hot Start DNA Polymerase	0.5	
Sample	5	-

Aliquot **20 µl** of each MM to desired wells on a 96-well plate or to PCR tubes in the PCR cabinet.

Add **5 µl** of cDNA to the designated wells in the sample addition cabinet.

Do this once for the Pool 1 MM and once for the Pool 2 MM.

Transfer samples to a thermal cycler and incubate using the following program:

A	B	C
Step	**Temperature**	**Time**
Heat Activation	95°C	30 seconds
40 Cycles	95°C	15 seconds
	63°C	5 minutes
Final Extension	72°C	2 minutes
Hold	4°C	

Set up for Fragmenation and Hybridization

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4

Preheat a hybridization oven to **45 °C**

Prewarm two thermal cyclers to **37 °C** and **95 °C** .

Resuspend lyophilized DNase I (Sigma #D4263):

Add **2 mL** of ice cold 1x DNase I Buffer to a vial of lyophilized DNase I.

Important! To avoid loss of material when opening the vial, gently tap the bottom of the vial before opening. Open slowly and be certain not to touch the stopper to the bench.

Reseal the vial with the stopper and invert 10 times to thoroughly mix.

Incubate for 30 minutes on ice to ensure full resuspension.

Invert an additional 10 times.

Note: store aliquots of resuspended enzyme at - **-20 °C** °C for up to 3 days. Do not re-freeze aliquots after thawing. Discard enzyme after use.

Prepare a fresh 500x dilution of DNase I using room temperature 1x DNase I buffer:
Add **40 µl** of resuspended enzyme to **20 mL** of room temperature 1xDNase I buffer.
Invert ten times to mix. Pour into a reagent reservoir.

Prepare 4x Hybridization buffer for use with a multichannel pipette - either aliquot to PCR tubes or add to a reagent reservoir.

5 **Fragment and Hybridization** 55m

Important! Fragmentation is highly time sensitive. A multichannel is highly recommended so that reactions can be started and stopped for all samples ~simultaneously.

Combine **20 µl** of the Pool 1 + **20 µl** of the Pool 2 RT-PCR products for each of the starting samples in a new PCR tube or plate.

Ensure samples are sealed. Gently vortex and spin.

Prewarm samples at **37 °C** for **00:05:00**

Add **40 µl** of 500x DNase I to samples. Vortex and briefly centrifuge samples.

Incubate for **00:45:00** at **37 °C**

Important! This step is highly time sensitive. Start your timer as soon as the enzyme is added to the samples.

To stop reactions, add **27 µl** of 4x ARTIC Hybridization Buffer. Vortex and briefly samples.

Incubate for **00:05:00** at **95 °C**

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Snap cool samples on ice.

Seal, vortex, and briefly centrifuge samples.

6 Samples can be stored at **-20 °C** at this point. If so, the heat denaturation (**95 °C**)and snap cool steps above should be repeated for best results.

7 Hybridize fragmented PCR product to QuadCore™ Microarray

Transfer **60 µl** of each sample to the desired wells of the hybridization tray.

Important! The well capacity of the 96-well hybridization tray is 40-70µL.
Hybridization relies on the target concentration in solution, not the total volume. If the full sample volume cannot be transferred without avoiding bubbles, do not attempt to transfer the full volume.
Optional: fill surrounding wells with 4xSSC to prevent evaporation.

Transfer the QuadCore™ chip plate to the tray and transfer the plate to **45 °C** .

Incubate at **45 °C** for 2 hours or overnight.

Note: longer incubation times of 16-24 hours will provide higher quality scores for base calling, however shorter incubation times of 1-6 hours can be used.

8 Wash/Stain

25m

Preheat a hybridization oven to **30 °C**

Prepare staining master mixes. Store on ice, dark:

A	B	C
SA Stain Reagent	**Volume (uL): total = 50µL**	**2x Number of Samples + 20%**
Nuclease-free water	37	
20x SSC	12.5	
Cy3-Streptavidin (1mg/mL)	0.5	

A	B	C
Anti-SA Stain Reagent	**Volume (uL): total = 50µL**	**Number of Samples + 10%**
Nuclease-free water	42.3	
10x PBS	5	
BSA (20mg/mL)	2.5	
Biotinylated Goat anti-SA	0.2	

After hybridization, transfer the chip plate to a wash tray containing 20 mL of Wash A ifor 00:01:00 at room temperature

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Transfer the chip plate to a wash tray containing **20 mL** of Wash B and transfer to **30 °C** for **00:05:00** .

Pipette **50 µl** of SA Stain MM into desired wells of a staining tray. Transfer the chip plate to this tray.

Incubate for **00:02:00** at room temperature, dark.

Note: staining may be improved by the use of an orbital shaker.

Transfer the chip plate to a wash tray containing **20 mL** of Wash A for **00:02:00** at room temperature.

Pipette **50 µl** of Anti-SA Stain MM into desired wells of a staining tray. Transfer the chip plate to this tray.

Incubate for **00:10:00** at room temperature, dark.

Transfer the chip plate to a wash tray containing **20 mL** of Wash A for **00:02:00** at room temperature.

Pipette **50 µl** of SA Stain MM into desired wells of a staining tray. Transfer the chip plate to this tray.

Incubate for **00:02:00** at room temperature, dark.

Transfer the chip plate to a wash tray containing **20 mL** of Scan Buffer for **00:01:00** at room temperature.

9 Image on Summit™ Instrument

Image on the Centrillion Technologies Summit™ Scanner equipped with the VirusHunter™ software, using three acquisition times (0.25, 1, and 4 seconds)
Important! QuadCore™MicroarraysProcessed withthe ARTIC-SARS-CoV-2-01 protocol will yield the best results if imaged over multiple acquisition times.

Initialize the software: double click the “Magpie” icon on the desktop to run the magpie program.

Select the location to store the files, and press “OK” button。

Right-Click the “Magpie” icon on the right lower of desktop and select the “E-Tools” option.

Open the lid of the Summit Scanner and place the scan tray into the machine. Ensure that the tray is flat and secure.
Important: Never insert a wet scan tray into the Summit™ scanner

Add **2 mL** of Scan Buffer to the scan tray.
Important! Avoid buffer leakage in the Summit™ at all costs! By adding buffer to the scan tray after it is in the holder, you limit the chance of leakage during the placement of the scan tray in the holder. Do not overfill the scan tray.

Add your plate with the RFID facing away.
Important! Be very careful to insert the plate in the correct orientation with the RFID in the back and the notch at the front left corner. The “Scanning Tray” label should be visible.

Close the lid.

Click the "plate in" button to enable reading of the RFID.

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Select your scanning channels and your chip numbers.

If you would like to have preview open while scanning, click preview before starting the scan.

Click "Scan."

10 Analyze data using the VirusHunter™ software

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