

SARS-COV-2 NGS ASSAY

FOR EMERGENCY USE ONLY

Instructions for Use

Catalog #102997, 96 reactions

For *In Vitro* Diagnostic Use

For Use Under Emergency Use Authorization (EUA) Only

For Prescription Use Only

IVD

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INTENDED USE

The SARS-CoV-2 NGS Assay is a next-generation sequencing (NGS) *in vitro* diagnostic test on the Illumina NextSeq 500, NextSeq 550, and NextSeq 550Dx Sequencing System intended for the qualitative detection of the SARS-CoV-2 RNA from nasopharyngeal (NP) swabs, oropharyngeal (OP) swabs, anterior nasal swabs, mid-turbinate nasal swabs, nasopharyngeal wash/aspirates, nasal wash/aspirates, and bronchoalveolar lavage (BAL) specimens that are collected from individuals suspected of COVID-19 by their healthcare provider.

Results are for the detection of the SARS-CoV-2 RNA. The SARS-CoV-2 RNA is generally detectable in respiratory specimens during the acute phase of infection. Positive results indicate the presence of the SARS-CoV-2 RNA; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Positive results do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all results to the appropriate health authorities.

Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

The SARS-CoV-2 NGS Assay is also intended for the identification and differentiation of SARS-CoV-2 Phylogenetic Assignment of Named Global Outbreak (PANGO) lineages and the identification of specific SARS-CoV-2 genomic mutations, when clinically indicated, from SARS-CoV-2 positive samples identified using the SARS-CoV-2 NGS Assay. It is intended to be used in conjunction with patient history and other diagnostic findings, when clinically indicated, i.e., in situations where results may aid in determining appropriate clinical management. Results of this test regarding the PANGO lineage and specific SARS-CoV-2 genomic mutations are intended to be interpreted by the ordering health care professional. Results should not be used as the sole basis for treatment or other patient management decisions.

Testing is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet requirements to perform high complexity tests. The SARS-CoV-2 NGS Assay is intended for use by qualified and trained clinical laboratory personnel specifically trained in the use of the Illumina NextSeq 500, NextSeq 550, and NextSeq 550Dx Sequencing System and Next Generation Sequencing workflows as well as *in vitro* diagnostic procedures. The SARS-CoV-2 NGS Assay is only for use under the Food and Drug Administration's Emergency Use Authorization.

PRINCIPLES OF THE PROCEDURE

The SARS-CoV-2 NGS Assay is a highly sensitive nucleic acid hybridization capture-based assay used for the detection and characterization of the SARS-CoV-2 virus. It utilizes Twist Bioscience's unique ability to rapidly develop virus-specific panels by DNA synthesis, and Biotia's comprehensive data analysis software and reporting capabilities (COVID-DX (v1)).

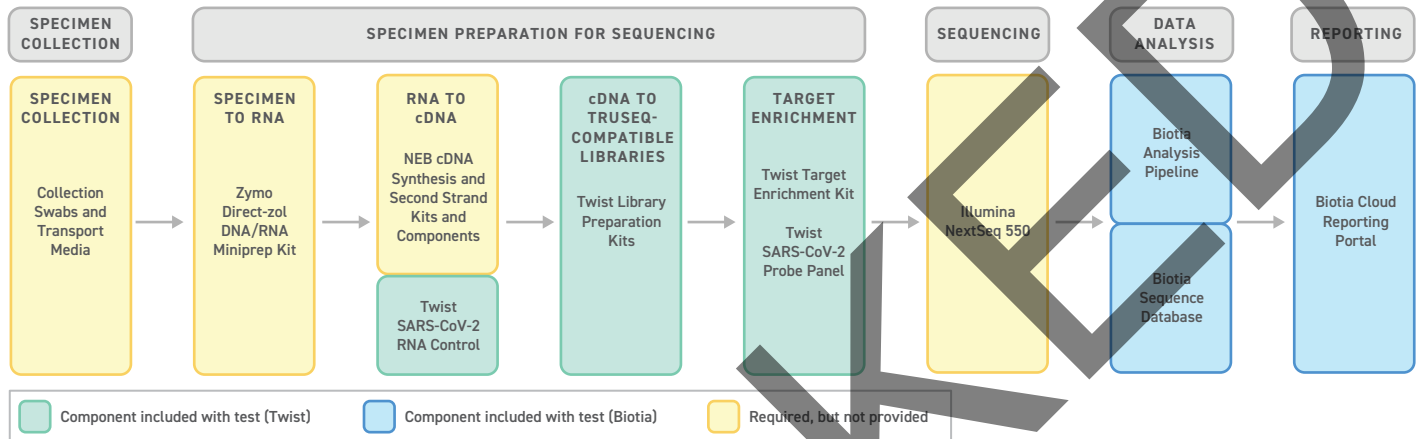


Figure 1: Workflow Overview Diagram. Components for the SARS-CoV-2 NGS Assay are specified as included by Twist (blue) or Biotia (green), and required but not provided (yellow). General sample and reagent processing steps are listed above (grey).

Panel Design

The panel used in this assay is designed to be 4x tiled against the SARS-CoV-2 reference genome (MN908947.3). It contains 994 probes that target the entire viral genome, spanning the length of about 30,000 bp. Although this panel is designed to target one strain of the genome, it is designed to enrich all strains by using conserved regions in the mutated viral genomes found in patient samples.

Specimen Collection, Specimen Preparation and Sequencing

The dsDNA biotin-bound hybrid capture panel used in the assay is designed to enrich for all evolved virus sequences in nasopharyngeal (NP) swabs, oropharyngeal (OP) swabs, anterior nasal swabs, mid-turbinate nasal swabs, nasopharyngeal wash/aspirates, nasal wash/aspirates, and bronchoalveolar lavage (BAL) specimens from patients with symptoms indicative of COVID-19. This capture-based assay utilizes cDNA synthesis from template viral and human RNA sequence in a patient's specimen, producing amplified libraries from the cDNA using a PCR thermocycler. The SARS-CoV-2 virus sequence is then targeted during a hybridization reaction with a biotin-attached probe specific to the SARS-CoV-2 virus. Targeted regions are extracted from the non-targeted regions using biotin-streptavidin chemistry and stringency washes. Remaining molecules are amplified using a PCR thermocycler and sequenced by an Illumina-based NGS platform (NextSeq 500, NextSeq 550, and NextSeq 550Dx system).

Data Analysis and Reporting

The Biotia COVID-DX (v1) software analyzes sequencing results to provide a clinical diagnostic report that includes the presence of the SARS-CoV-2 virus in each sample, for diagnostic use under the FDA Emergency Use Authorization. If additionally prescribed, the software will also provide a variant report that includes the PANGO lineage designation and information on identified genetic mutations for positively identified samples. Access to the Biotia COVID-DX (v1) software, data analysis and report generation will be provided through the clinician's unique login with credits included after each SARS-CoV-2 NGS Assay purchased.

DESCRIPTION AND EXPLANATION

Specimen Collection

Clinical specimens will be collected following the current CDC guidelines [cdc.gov/coronavirus/2019-ncov/lab/guidelines-clinical-specimens.html](https://www.cdc.gov/coronavirus/2019-ncov/lab/guidelines-clinical-specimens.html). The manufacturer instructions for specimen collection will be followed. Specimens will immediately be placed into sterile tubes containing 2–3 ml of viral transport media. Transportation of patient samples must comply with all applicable governing regulations for the transport of etiological agents. Specimens are stable for up to 24 hours at room temperature or up to 72 hours when stored at 2°C to 8°C but should be transported and tested as soon as possible after collection. If specimens cannot be tested within 72 hours of collection, they should be frozen at –70°C or colder until tested.

Specimen to RNA

Patient RNA is isolated and purified from each specimen by manual extraction using Zymo's Direct-zol DNA/RNA MiniPrep kit (spin column, 100 µg binding capacity). The input volume for extraction is 250 µl, and the nucleic acid elution volume is 50 µl for extracted RNA.

RNA to cDNA

Extracted and purified patient RNA samples are converted to cDNA through random priming using NEB's Random Primer 6, ProtoScript II First Strand cDNA Synthesis kit, and NEBNext Ultra II Non-Directional RNA Second Strand Synthesis kit reagents.

cDNA to TruSeq-Compatible Libraries

The cDNA samples are converted to Illumina TruSeq-compatible libraries. This is done using the Twist Library Preparation kit and the Enzymatic Fragmentation with Unique Dual Indices. Each library will be quantified to determine size and concentration by a TapeStation System (Agilent Technologies) and a Qubit Fluorometer (Thermo Fisher). For volumes and thermocycling conditions related to the library generation process, please see below. Each index has a unique 10 bp barcode that is identified by the user and during the sequencing process. The sequences for each primer used in this process, including the barcode, can be found in the EUA Supplementary Information spreadsheet. There are 96 total barcodes available for use. After library generation, pooling occurs using 8 uniquely barcoded libraries per pool, to create 12 distinct 8-plex reactions.

Target Enrichment

Hybrid capture is performed using 187.5 ng of library in each 8-plex hybridization reaction (1,500 ng total), using a 2-hour Twist Fast Hybridization time. The biotin-bound panel used to enrich, called the Twist SARS-CoV-2 Research Panel, targets libraries containing the SARS-CoV-2 virus. All sequences that make up the Twist SARS-CoV-2 Research Panel can be found in the EUA Supplementary Information spreadsheet under tab titled "Section G, Part 1.1". The panel was designed against the SARS-CoV-2 reference genome (MN908947.3), which is the Wuhan-Hu-1 isolate genome. Libraries captured in this process are isolated from human libraries using biotin/streptavidin bead chemistry. Beads are washed several times to reduce the number of human libraries in the sequencing pool.

Illumina Sequencing

All 12 enriched library pools are sequenced with 150 bp single-end reads on the Illumina NextSeq platform using a NextSeq500/550 High Output kit. Due to the nature of the multiplexing and uniquely barcoded sequences, all 96 samples can be sequenced on one run, with 1M reads per sample.

Biotia Analysis Pipeline

Data will be analyzed using a cloud-based Biotia COVID-DX (v1) software that has been optimized for the SARS-CoV-2 NGS Assay. The analysis pipeline includes the following major steps with the cutoffs described:

1. Removal of low-quality reads based on a minimum read length of 20 bp.
2. Down sampling of the FASTQs to a maximum of 12,500,000 reads per FASTQ to cap computational runtime.
3. Alignment to reference viral and host genomes. The purpose of this step is to filter out reads that cannot be mapped specifically to SARS-CoV-2. This reduces the probability of laboratory cross-contamination and / or cross-hybridization of the probes influencing results. Reference viral and host genomes include:

SARS-CoV-2 (NC_045512.2), Human genome (GrC38), Adenovirus (NC_001405.1), Human Metapneumovirus (hMPV; NC_039199.1), Parainfluenza virus 1 (JQ901971.1), Parainfluenza virus 2 (NC_003443.1), Parainfluenza virus 3 (NC_001796.2), Parainfluenza virus 4 (KF483663.1), Influenza A (AB284320.1), Influenza B (NC_002208.1), Enterovirus (AY426531.1), Respiratory syncytial virus (NC_038235.1), Rhinovirus (L24917.1), Haemophilus influenzae (ATCC_51907), Legionella pneumophila (ATCC_33152), Mycobacterium tuberculosis (NC_000962.3), Streptococcus pneumoniae (ATCC_700669), Streptococcus pyogenes (ATCC_12344), Mycoplasma pneumoniae (NZ_CP010546.1), Candida albicans (CP017623-30.1), Pseudomonas aeruginosa (ATCC_9027), Staphylococcus epidermidis (ATCC_12228), Streptococcus salivarius (ATCC_9759), Human coronavirus 229E (NC_002645.1), Human coronavirus OC43 (NC_006213.1), Human corona- virus HKU1 (NC_006577.2), Human coronavirus NL63 (DQ445911.1), MERS-coronavirus (KT006149.2), Chlamydia pneumonia (NC_005043.1), Pneumocystis jirovecii (PCP; GCA_001477535.1), Bordetella pertussis (CP011448.1).
4. Extraction of reads mapped exclusively to NC_045512.2.
5. Calculation of coverage and hybrid selection metrics across NC_045512.2 and GrC38.
6. Calculation of the validity of the sample. If the sample is a test sample or positive control, it must have at least 10,000 reads post minimum read length filtering and at least 10,000 on-target bases within GrC38 to be deemed valid.
7. Calculation of the integral under the coverage curve of NC_045512.2 to determine presence or absence of SARS-CoV-2. The minimum integral threshold for samples with less than or equal to 10,000 on-target bases is 8.6. The minimum integral threshold for samples with greater than 10,000 on-target bases is 9.5.

Lineage is determined using Pangolin (minimum v4.0.3) via PangoLEARN (minimum version 04/15/2022). Genetic mutations are determined by aligning the test strain to the SARS-CoV-2 reference genome (NC_045512.2) with information on gene location/site and alteration.

Biotia Cloud Reporting Portal

The Biotia COVID-DX (v1) software will provide two separate clinical reports including presence/absence of the SARS-CoV-2 RNA and its genetic mutations. Access to the Biotia COVID-DX (v1) software, data analysis and report generation will be provided through the clinician's unique login, with credits included after each SARS-CoV-2 NGS Assay purchased.

Each test is accompanied by a unique order number and test ID throughout the entire process. These two identifiers are required to reconnect the test results to the correct patient.

SPECIAL INSTRUMENT REQUIREMENTS

The SARS-CoV-2 NGS Assay is to be used with a manual extraction protocol, the Illumina NextSeq 500, NextSeq 550, or NextSeq 550Dx sequencer instruments, the Agilent TapeStation (QC) and cloud-based Biotia COVID-DX (v1) Software to translate genetic data to clinical reports. The following instruments are required in order to perform the SARS-CoV-2 NGS Assay workflow:

INSTRUMENTS	SUGGESTED SUPPLIER	PART NUMBER
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific	Q33216
4200 TapeStation System	Agilent Technologies	G2991AA
Local Run Manager Generate FASTQ Analysis Module v2	Illumina	Download at support.illumina.com/sequencing/sequencing_software/local-run-manager/downloads.html
NextSeq 500 or NextSeq 550 or NextSeq 550Dx	Illumina	SY-415-1001 SY-415-1002 20005715
Thermomixer for 1.5 ml tubes (2x)	Eppendorf	223100667
Thermocycler	VWR	89235-826

MATERIALS PROVIDED

Components manufactured by Twist Bioscience or Biotia and supplied with the test can be ordered via top fill product number #102997 (for 96 reactions) and include:

PRODUCT NUMBER (MANUFACTURER)	TEST KIT NAME	QUANTITY	REAGENT NAME	VOLUME (ML)	STORAGE
102875 (Twist)	Twist Synthetic SARS-CoV-2 RNA Control 2 (MN908947.3)	1	Twist Synthetic SARS-CoV-2 RNA Control 2 (MN908947.3)	0.10	-90 to -70°C
102874 (Twist)	Twist Library Preparation Kit 2	2	DNA Purification Beads	12.50	2-8°C
102873 (Twist)	Twist Library Preparation EF Kit 1	1	Fragmentation Enzyme	0.96	-25 to -15°C
			Fragmentation Buffer	0.48	-25 to -15°C
			DNA Ligation Mix	0.96	-25 to -15°C
			DNA Ligation Buffer	1.90	-25 to -15°C
			DNA Purification Beads	12.50	2-8°C
102876 (Twist)	Twist Universal Adapter System - Plate A	1	Twist Universal Adapters - TruSeq Compatible	0.48	-25 to -15°C
			Twist UDI Primers - TruSeq Compatible, Plate A	0.01 (per primer set)	-25 to -15°C
102880 (Twist)	Twist SARS-CoV-2 Research Panel	1	Twist SARS-CoV-2 Research Panel	0.384	-25 to -15°C
102881 (Twist)	Twist Universal Blockers	1	Blocker Solution	0.48	-25 to -15°C
			Universal Blockers	0.77	-25 to -15°C
102882 (Twist)	Twist Binding and Purification Beads	1	DNA Purification Beads	8.60	2-8°C
			Streptavidin Binding Beads	9.60	2-8°C
102883 (Twist)	Twist Fast Hybridization Reagents	1	Fast Hybridization Mix	2.30	-25 to -15°C
			Amplification Primers, ILMN	0.24	-25 to -15°C
			Hybridization Enhancer	2.90	-25 to -15°C
102884 (Twist)	Twist Fast Wash Buffers	1	Fast Binding Buffer	77.00	2-8°C
			Fast Wash Buffer 1	43.00	2-8°C
			Wash Buffer 2	67.00	2-8°C
104270 (Biotia)	Biotia COVID-DX (v1)	1	N/A	N/A	N/A

MATERIALS REQUIRED BUT NOT PROVIDED

Components required but not included with the test:

REAGENT/CONSUMABLE	MANUFACTURER	PRODUCT NUMBER
Direct-zol DNA/RNA Extraction Kit	Zymo Research	R2081
ProtoScript II First Strand cDNA Synthesis Kit	New England Biolabs	E6560L
NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module	New England Biolabs	E6111
Random Primer 6	New England Biolabs	S12305
Gene Expression Universal Reference RNA (Human)	Agilent Technologies	740000-41
VERO C1008 [Vero 76, clone E6, Vero E6]	ATCC	CRL-1586
Viral Transport Media (VTM)	N/A	N/A
KAPA HiFi HotStart ReadyMix	Kapa Biosystems	KK2602
Ethanol (200 proof)	N/A	N/A
Nuclease-free water	N/A	N/A
Qubit RNA High Sensitivity Quantification Assay	Thermo Fisher Scientific	Q32855
Qubit dsDNA Broad Range Quantitation Assay	Thermo Fisher Scientific	Q32853
Qubit dsDNA High Sensitivity Quantitation Assay	Thermo Fisher Scientific	Q32854
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific	Q33216
D5000 DNA Screen Tape	Agilent Technologies	5067-5588
D5000 Reagents	Agilent Technologies	5067-5589
D1000 DNA Screen Tape	Agilent Technologies	5067-5582
D1000 Reagents	Agilent Technologies	5067-5583
D1000 High Sensitivity Screen Tape	Agilent Technologies	5067-5584
D1000 High Sensitivity Reagents	Agilent Technologies	5067-5585
4200 TapeStation System	Agilent Technologies	G2991AA
NextSeq 500/550 High Output Kit v2.5 (150 Cycles)	Illumina	20024907
NextSeq PhiX Control Kit	Illumina	FC-110-3002
1M Sodium Hydroxide	Teknova	H0224
NextSeq 500 or NextSeq 550 or NextSeq 550Dx	Illumina	SY-415-1001 SY-415-1002 20005715
Local Run Manager Generate FASTQ Analysis Module v2	Illumina	Download at support.illumina.com/sequencing/sequencing_software/local-run-manager/downloads.html
Thermomixer for 1.5 ml tubes (2x)	Eppendorf	2231000667
Centrifuge with 96-well plate rotor	N/A	N/A
Centrifuge for 1.5 ml tubes	N/A	N/A
Thermal cycler (96-well) with heated lid	VWR	89235-826
SpeedVac vacuum concentrator with plate rotor	N/A	N/A
2.0 ml microcentrifuge tubes	Eppendorf	022431048
1.5 ml microcentrifuge tubes	Eppendorf	022431021
Thin-walled PCR 0.2 ml strip-tubes	Eppendorf	951010022
96-well thermal cycling plates	Applied Biosystems	N8010560

CONTINUED ON THE NEXT PAGE

REAGENT/CONSUMABLE	MANUFACTURER	PRODUCT NUMBER
Adhesive PCR plate foils	Agilent Technologies	5067-5154
PCR cryogenic rack	N/A	N/A
Pipettes	N/A	N/A
Pipette tips	N/A	N/A
Vortex mixer	N/A	N/A
Benchtop mini centrifuge for 1.5 ml tubes	N/A	N/A
1.5 ml compatible magnetic stand	Invitrogen	12321D
96-well compatible magnetic plate	Invitrogen	12331D

REVOKED

GENERAL WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic use (IVD) only.
- For prescription use only.
- For use under Emergency Use Authorization (EUA) only.
- This product has not been FDA cleared or approved, but has been authorized for emergency use by FDA under an EUA for use by authorized laboratories.
- This product has been authorized only for the detection of nucleic acid from SARS-CoV-2, along with the identification and differentiation of SARS-CoV-2 PANGO lineages and the identification of specific SARS-CoV-2 genomic mutations, not for any other viruses or pathogens.
- The emergency use of this product is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of *in vitro* diagnostic tests for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Act, 21 U.S.C. § 360bbb-3(b)(1), unless the declaration is terminated or authorization is revoked sooner.
- Laboratories are required to report all results to the appropriate public health authorities.
- This assay contains potentially hazardous chemicals. For health and safety information, review Safety Data Sheets (SDS) available on the Twist Bioscience website prior to handling any chemical materials.
- Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Use in a well-ventilated area, wear protective clothing, and dispose of any containers and unused contents in accordance with applicable local governmental safety standards.
- This assay contains a flammable chemical. Keep away from heat and open flames. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Use in a well-ventilated area, wear protective clothing, and dispose of any containers and unused contents in accordance with applicable local governmental safety standards.
- Handle in accordance with good industrial hygiene and safety practices. Wash after handling this material and before eating, drinking, and/or smoking. Regular cleaning of equipment, work area, and clothing is recommended.
- Use routine laboratory precautions. Do not pipette by mouth. Do not eat, drink, or smoke in designated work areas. Wear disposable gloves and laboratory coats when handling specimens and assay reagents. Wash hands thoroughly after handling specimens and assay reagents.
- Do not reuse containers. Dispose of containers in accordance with applicable regional, national, and local laws and regulations.
- Do not use any assay components beyond their stated expiration date on the assay box label. Do not interchange assay components from different assay lots. Assay lots are identified on the assay box label. Store the assay components at the specified temperature.
- To prevent sample or reagent degradation, make sure that all sodium hypochlorite vapors from cleaning have fully dissipated before starting the protocol.
- Failure to follow the procedures as outlined can result in erroneous results or significant reduction in sample quality.

Avoiding Contamination:

- Use proper laboratory practices to prevent contamination of PCR products by nucleases, etc., as it could lead to inaccurate and unreliable results.
- Perform library preparation in a RNase/DNase-free environment. Thoroughly decontaminate work areas with a RNase/

DNase-inhibiting solution.

- Use fresh tips and consumable labware between samples and dispensing reagents.
- Use aerosol-resistant tips to reduce the risk of carry-over and sample-to-sample cross-contamination.
- To prevent contamination, take extreme care to make sure that well contents remain fully in the well. Do not splash contents.
- Do not use aerosol bleach sprays when performing library preparation. Trace bleach contamination can lead to assay failure.
- Use a unidirectional workflow when moving from pre-amplification to post-amplification environments.
- Carefully adhere to the procedures specified in this package insert. Due to the high capture efficiency of the SARS-CoV-2 NGS Assay, extremely low levels of contamination between samples can be seen in the negative control results.
- Always handle negative control RNA or cDNA template first during sample generation.
- Use the same set of Twist UDI barcoded primer(s) in each end-to-end workflow for your control(s).
- After each incubation or thermal cycling step, make sure to spin down plates and tubes to avoid cross-contamination from evaporated droplets generated during the process.
- Remove aluminum plate covers carefully and change gloves after doing so.
- **All workstation surfaces, benchtop equipment, pipettes, and gloves should be thoroughly cleaned prior to each use with 10% bleach solution, 70% ethanol, and RNase Away®. This is done to avoid contamination between samples and from outside sources.**

REAGENT STORAGE, HANDLING, AND STABILITY

- Room temperature is defined as 15°C to 30°C.
- Store all RNA samples at –85°C to –75°C when not in use. Keep on ice at all times when in use.
- Store all kit components and reagents at the appropriate temperature, as labeled on each kit or tube label.
- Avoid multiple freeze-thaw cycles with reagents and samples.
- All purification resuspensions should be done in nuclease-free water.
- Always inspect expiration dates on each kit prior to use. Do not use expired reagents.
- Primers, panels, reagents, controls, and enzymes must be thawed and kept on a cold block or ice at all times during preparation and use.

SPECIMEN COLLECTION, HANDLING, AND STORAGE

Inadequate or inappropriate specimen collection, storage, and transport are more likely to yield false test results. Training in specimen collection is highly recommended due to the importance of specimen quality. Handle all specimens as you would infectious agents.

Collecting Specimens

- Refer to Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Patients Under Investigation (PUIs) for 2019 Novel Coronavirus (2019-nCoV) [cdc.gov/coronavirus/2019-nCoV/guidelines-clinical-specimens.html](https://www.cdc.gov/coronavirus/2019-nCoV/guidelines-clinical-specimens.html)
- Follow specimen collection device manufacturer instructions for proper collection methods.
- Nasopharyngeal specimen samples should be collected using only swabs with a synthetic tip, such as nylon or Dacron®, and an aluminum or plastic shaft. Calcium alginate swabs are unacceptable and cotton swabs with wooden shafts are not recommended. Place swabs immediately into sterile tubes containing 2–3 ml of viral transport media.

Transporting Specimens

- Transportation of patient samples must comply with all applicable governing regulations for the transport of etiologic agents.

Storing Specimens

- Specimens should be transported and tested as soon as possible after collection.
- Specimens are stable for up to 24 hours at room temperature or up to 72 hours when stored at 2°C to 8°C. If specimens cannot be tested within 72 hours of collection, they should be frozen at –70°C or colder until tested.

REAGENT AND CONTROLS PREPARATION

- For concentration quantification, use the Thermo Fisher Scientific Qubit Quantitation Assay to quantify input purified cDNA. Measuring cDNA concentration by absorbance at 260 nm is not recommended.
- No cations and chelators should be added to the system after cDNA synthesis. The presence of cations and chelators may affect the initial fragmentation reaction.
- The library preparation step may yield more material than needed for target enrichment. Excess product can be stored at -20°C for later use.
- The Twist Fast Hybridization Mix is viscous. Pipette slowly to ensure accurate pipetting. Additional considerations include using a wide-bore or larger volume pipette tip.
- Small white particles may be present in the Twist SARS-CoV-2 Research Panel. This will have no effect on the final capture product.
- Twist Hybridization Enhancer will settle as a top layer in the hybridization reaction after the final pulse-spin. This is normal and will have no effect on the final capture product.
- Rapid transfer directly from the thermal cycler at 60°C is a critical step to achieve a low off-target rate. Do not remove the tube(s) of the hybridization reaction from the thermal cycler or allow it to cool to less than 60°C before transferring the solution to the washed Twist Streptavidin Binding Beads.
- Salts may precipitate out of the Twist Fast Binding Buffer, Twist Fast Wash Buffer 1 and Twist Wash Buffer 2 when solutions are stored. Preheating each buffer helps to bring these salts back into solution.
- If the available sequencing platform to be used is an Illumina NextSeq 550Dx, please use the platform in RUO mode.

MODULE 1 NUCLEIC ACID EXTRACTION

GUIDELINES

Prepare in advance the extraction reagents and extraction controls as follows:

Controls:

- For the negative extraction control (NEC), aliquot 20 μl of ATCC VeroE6 cells ($\sim 4.0\text{--}4.5 \times 10^4$ cells total) into 2 ml tubes containing 230 μl of VTM. Store aliquots at -80°C until ready for use.
- For the negative template control (NTC), aliquot 250 μl of VTM into 2.0 ml tubes. Store aliquots at room temperature until ready for use.

Reagents:

- Before using the Zymo Direct-zol DNA/RNA Miniprep kit, add 48 ml of 100% ethanol to the 12 ml of Zymo RNA Wash Buffer concentrate.

STAGE 1: NUCLEIC ACID EXTRACTION

- 1.1.1** Aliquot 250 μl of the clinical specimen into a 2 ml microcentrifuge tube. Additionally, use one aliquot of both the negative extraction control and negative template control per each extraction run.
- 1.1.2** Add 650 μl of Zymo TRI Reagent to each sample and briefly vortex.
- 1.1.3** Incubate samples for 1 hour at room temperature.
- 1.1.4** Add 900 μl of 100% ethanol and mix thoroughly.
- 1.1.5** Transfer 700 μl of the mixture into a Zymo-Spin™ IICR Column in a Collection Tube and centrifuge at $16,000 \times g$ for 30 seconds. Adequately discard flow-through and repeat this step until all of the sample has been loaded into the column. Transfer the column into a new collection tube.
- 1.1.6** Add 400 μl of Zymo RNA Prep Buffer to the column and centrifuge at $16,000 \times g$ for 30 seconds. Discard the flow-through and repeat this step.
- 1.1.7** Add 700 μl of Zymo RNA Wash Buffer to the column and centrifuge for 1 minute at $16,000 \times g$ to ensure complete removal of the wash buffer. Carefully transfer the column into a 1.5 ml nuclease-free tube.
- 1.1.8** To elute RNA, add 50 μl of nuclease-free water directly to the column matrix and centrifuge at $16,000 \times g$ for 30 seconds.
- 1.1.9** Place samples on ice and proceed to QC the extracted samples by quantifying RNA with Qubit RNA High Sensitivity Quantification Assay. Please see the “Quality Control Procedures” section for details.

THIS IS A SAFE STOPPING POINT.

STORE SAMPLES AT -80°C IF NOT IMMEDIATELY PROCEEDING TO MODULE 2.

MODULE 2 cDNA SYNTHESIS

GUIDELINES

Prepare the cDNA synthesis reagents and controls in advance (the positive and internal controls) as follows:

Controls:

- Dilute the Agilent Gene Expression Universal Reference RNA (Human) to 3.85 ng/μl.
- Universal Human Reference RNA is provided in a solution of 70% ethanol and 0.1 M sodium acetate. Prepare the Reference RNA for use as follows:
 - Centrifuge the tube at 12,000 × g for 15 minutes at 4°C.
 - Carefully remove the supernatant.
 - Wash the pellet in 70% ethanol.
 - Centrifuge the tube at 12,000 × g for 15 minutes at 4°C.
 - Carefully remove the supernatant and air-dry the pellet at room temperature for 30 minutes to remove retained ethanol.
 - Resuspend the pellet by adding 1 ml of nuclease-free water for a final concentration of 200 ng/μl. Vortex thoroughly and quick spin.
 - Finally, add 9.6 μl of the Human Reference at 200 ng/μl to 490.4 μl of nuclease free water. Vortex thoroughly and spin down. Aliquot this final dilution into 10 tubes of 50 μl. Freeze all aliquots at –80 °C and thaw as needed for each run.
- Aliquot 13 μl of the diluted Agilent Gene Expression Universal Reference RNA (Human) into 0.5 ml tubes.
- Dilute the Twist Synthetic SARS-CoV-2 RNA Control 2 (MN908947.3) to 96 copies/μl.
- To dilute the synthetic control, perform a series of serial dilutions in order to get the control at 96 copies/μl concentration (3.2x of the LoD). First, do a 1/10 dilution of the synthetic control by adding 5 μl of the control in 45 μl of nuclease free water in a 1.5 ml tube. Vortex thoroughly and spin down. This dilution would be at 100,000 copies/μl. Subsequently, add 5 μl of the freshly diluted control into 495 μl of nuclease free water. Vortex thoroughly and spin down. This intermediate dilution would be at 1,000 copies/μl. Finally, dilute the control to a final concentration of 96 copies/μl by adding 9.6 μl of the 1,000 copies/μl dilution to 90.4 μl of nuclease free water. Vortex thoroughly and spin down. Aliquot this final dilution into 2 μl aliquots. Freeze all aliquots at –80°C and thaw as needed for each run.
- For the positive control (PC): add 2 μl of diluted Twist Synthetic SARS-CoV-2 RNA Control 2 (MN908947.3) to 0.5 ml tubes containing 13 μl of the diluted Agilent Gene Expression Universal Reference RNA (Human).
- For the internal control (IC), add 2 μl of nuclease-free water to 0.5 ml tubes containing 13 μl of the diluted Agilent Gene Expression Universal Reference RNA (Human).
- Freeze aliquots at –80°C and thaw out one aliquot of each control when ready to start the cDNA synthesis module.

INITIAL CONCENTRATION (COPIES/μl)	FINAL CONCENTRATION (COPIES/μl)	SYNTHETIC CONTROL (μl)	NUCLEASE FREE WATER (μl)
1e6	100,000	5	45
100,000	1,000	5	495
1,000	96	9.6	90.4

Reagents:

- Hydrate each tube of lyophilized NEB Random Primers (18.6 nmoles) to a 50 μM concentration by adding 372 μl of nuclease-free water. Mix by vortexing.
- Aliquot 500 μl of 50 μM NEB Random Primers into a 1.5 ml tube. Store aliquots at -20°C until ready for use.

STAGE 1: FIRST STRAND SYNTHESIS

2.1.1 On ice, thaw the RNA samples and the diluted PC and IC.

2.1.2 Additionally, thaw the NEB ProtoScript II First Strand cDNA Synthesis reagents, NEBNext Ultra II Non-Directional RNA Second Strand Synthesis reagents, and NEB Random Primers on ice.

2.1.3 Add the following volumes of reagents to each well to generate the primer annealing solution (either a thin-walled PCR 0.2 ml strip-tube or a 96-well thermal cycling plate).

REAGENT	VOLUME
50 μM NEB Random Primer 6	5 μl
RNA samples	15 μl
Total	20 μl

2.1.4 Close the strip tubes or seal the plate with an adhesive PCR plate foil. Mix by flicking the tube(s).

2.1.5 Pulse-spin the tube(s) and ensure there are minimal bubbles present.

2.1.6 Heat the primer annealing solution to 95°C for 5 minutes in a thermal cycler with the lid at 105°C , then ramp down to 20°C . Immediately place it on ice or on a PCR cryogenic rack. Proceed to the next step.

2.1.7 Prepare the first strand cDNA synthesis master mix with the following volumes of reagents per sample.

REAGENT	VOLUME
NEB ProtoScript II Reaction Mix	25 μl
NEB ProtoScript II Enzyme Mix	5 μl

2.1.8 Add 30 μl of first strand cDNA synthesis master mix to each sample, close the strip tubes or seal the plate with an adhesive PCR plate foil and mix by flicking the tube(s).

2.1.9 Pulse-spin the tube(s) and ensure there are minimal bubbles present.

2.1.10 Incubate the first strand synthesis solution in a thermal cycler as follows: 25°C for 5 minutes, 42°C for 1 hour, 80°C for 5 minutes with a heated lid at 105°C . When the incubation is finished, place samples on ice or a PCR cryogenic rack.

STAGE 2: SECOND STRAND SYNTHESIS

2.2.1 Prepare a second strand synthesis master mix with the following volumes per sample.

REAGENT	VOLUME
Nuclease-free water	18 μ l
NEBNext Second Strand Synthesis Reaction Buffer	8 μ l
NEBNext Second Strand Synthesis Enzyme Mix	4 μ l

2.2.2 Add 30 μ l of second strand synthesis master mix to each tube of first strand synthesis solution. Close the strip tubes or seal the plate with an adhesive PCR plate foil. Mix by flicking the tube(s).

2.2.3 Pulse-spin the tube(s) and ensure there are minimal bubbles present.

2.2.4 Incubate the second strand synthesis solution in a thermal cycler, with the heated lid off, at 16°C for 1 hour.

STAGE 3: cDNA CLEAN UP

2.3.1 While the second strand synthesis solution is incubating, equilibrate the Twist DNA Purification Beads at room temperature and prepare a fresh solution of 80% ethanol (500 μ l of per sample).

2.3.2 When the second strand synthesis solution incubation is finished, remove the reactions from the thermal cycler.

2.3.3 Vortex the pre-equilibrated Twist DNA Purification Beads until mixed.

2.3.4 Add 104 μ l (1.3x) of homogenized Twist DNA Purification Beads to each tube of second strand synthesis solution. Close the strip tubes or seal the plate with an adhesive PCR plate foil. Mix well by vortexing and pulse-spin.

2.3.5 Incubate the samples for 5 minutes at room temperature.

2.3.6 Place the samples on a magnetic plate for 1 minute or until supernatant is clear.

2.3.7 Without removing the plate or tubes from the magnetic plate or disturbing the bead pellet, remove and discard the supernatant.

2.3.8 Wash the bead pellet by gently adding 200 μ l of 80% ethanol (do not disturb the pellet), incubate for 1 minute, then remove and discard the ethanol.

2.3.9 Repeat this wash once, for a total of two washes, while keeping the samples on the magnetic plate.

2.3.10 Carefully remove all remaining ethanol with a 10 μ l pipet, making sure not to disturb the bead pellet.

- 2.3.11** _____ Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not over dry the bead pellet.
- 2.3.12** _____ Remove the plate or tubes from the magnetic plate and add 22 μ l of nuclease-free water to each sample. Mix by pipetting until homogenized.
- 2.3.13** _____ Incubate at room temperature for 2 minutes.
- 2.3.14** _____ Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.
- 2.3.15** _____ Transfer 20 μ l of the clear supernatant to a clean thin-walled PCR 0.2 ml strip-tube or well of a 96-well thermal cycling plate, without disturbing the bead pellet.
- 2.3.16** _____ Place samples on ice and proceed to QC the purified cDNA samples by quantifying with Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay. Please see the “Quality Control Procedures” section for details.

THIS IS A SAFE STOPPING POINT.

STORE SAMPLES AT -20°C IF NOT IMMEDIATELY PROCEEDING TO MODULE 3.

MODULE 3 DNA LIBRARY PREPARATION

STAGE 1: ENZYMATIC FRAGMENTATION, END REPAIR, AND dA-TAILING

3.1.1 Thaw the 5x Twist Fragmentation Enzyme and 10x Twist Fragmentation Buffer on ice. Mix 10x Twist Fragmentation Buffer by flicking the tube.

3.1.2 Program the thermal cycler with the conditions below. Set the lid temperature to 70°C. Start the program to pre-chill the thermal cycler.

	TEMPERATURE	TIME
1	4°C	HOLD
2	32°C	22 minutes
3	65°C	30 minutes
4	4°C	HOLD

3.1.3 Dilute the cDNA samples to 1 ng/μl with nuclease-free water. Mix well with gentle pipetting.

3.1.4 Add 25 μl of each diluted cDNA sample (25 ng total) into a thin-walled PCR 0.2 ml strip-tube or well of a 96-well thermal cycling plate, and place it on ice or a PCR cryogenic rack.

3.1.5 Pulse-spin to ensure all of the solution is at the bottom of the tube.

3.1.6 Prepare an enzymatic fragmentation master mix using the volumes listed below per sample. Mix well by gentle vortexing.

REAGENT	VOLUME PER REACTION
Nuclease-free water	10 μl
10x Twist Fragmentation Buffer	5 μl
5x Twist Fragmentation Enzyme	10 μl
Total	25 μl

3.1.7 Add 25 μl of enzymatic fragmentation master mix to each 25 μl cDNA sample well or tube, close the strip tubes or seal the plate with adhesive PCR plate foil. Mix well by vortexing.

3.1.8 Pulse-spin the sample plate or tubes and immediately transfer to the pre-chilled thermal cycler. Proceed to steps 2–4 of the thermal cycler program.

STAGE 2: TWIST UNIVERSAL ADAPTER LIGATION

3.2.1 While the fragmentation reaction is incubating, thaw the Twist Universal Adapters, Twist DNA Ligation Mix, and Twist DNA Ligation Buffer on ice. Additionally, equilibrate the Twist DNA Purification Beads at room temperature.

3.2.2 When the thermal cycler program is complete and the sample block has returned to 4°C, remove the samples from the block and place on ice or a PCR cryogenic rack.

3.2.3 Program a thermal cycler to incubate the samples at 20°C with the heated lid set to minimum temperature or turned off. Start the program so that the cycler is at 20°C while the samples are being prepared.

3.2.4 Add 2.5 µl of Twist Universal Adapters into each sample well or tube containing the dA-tailed DNA fragments. Mix gently by pipetting. Keep on ice.

3.2.5 Prepare a ligation master mix with the volumes indicated below. Mix well by gentle pipetting.

REAGENT	VOLUME PER REACTION
Nuclease-free water	17.5 µl
Twist DNA Ligation Buffer	20 µl
Twist DNA Ligation Mix	10 µl
Total	47.5 µl

3.2.6 Add 47.5 µl of the ligation master mix to the sample and mix well by gentle pipetting.

3.2.7 Seal or cap the tubes and pulse-spin to ensure all solution is at the bottom of the tube.

3.2.8 Incubate the ligation reaction at 20°C for 15 minutes in the thermal cycler, then move the samples to the bench top.

STAGE 3: LIGATION CLEAN UP

3.3.1 While the ligation reaction is incubating, thaw the KAPA HiFi HotStart ReadyMix and Twist UDI Primers on ice and prepare a fresh solution of 80% ethanol (500 µl per sample).

3.3.2 When the ligation reaction incubation is finished, remove the reactions from the thermal cycler.

3.3.3 Vortex the pre-equilibrated Twist DNA Purification Beads until mixed.

3.3.4 Add 80 µl (0.8x) of homogenized Twist DNA Purification Beads to each ligation reaction. Mix well by vortexing.

3.3.5 Incubate the samples for 5 minutes at room temperature.

- 3.3.6** _____ Place the samples on a magnetic plate for 1 minute or until supernatant is clear.
- 3.3.7** _____ Without removing the plate or tubes from the magnetic plate, remove and discard the supernatant.
- 3.3.8** _____ Wash the bead pellet by gently adding 200 µl of 80% ethanol (do not disturb the pellet), incubate for 1 minute, then remove and discard the ethanol.
- 3.3.9** _____ Repeat this wash once, for a total of two washes, while keeping the samples on the magnetic plate.
- 3.3.10** _____ Carefully remove all remaining ethanol with a 10 µl pipette, making sure not to disturb the bead pellet.
- 3.3.11** _____ Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not over dry the bead pellet.
- 3.3.12** _____ Remove the plate or tubes from the magnetic plate and add 17 µl of nuclease-free water to each sample. Mix by pipetting until homogenized.
- 3.3.13** _____ Incubate at room temperature for 2 minutes.
- 3.3.14** _____ Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.
- 3.3.15** _____ Transfer 15 µl of the clear supernatant to a clean thin-walled PCR 0.2 ml strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.
- 3.3.16** _____ Place the samples on ice or a PCR cryogenic rack.

STAGE 4: PCR AMPLIFICATION

- 3.4.1** _____ Program a thermal cycler with the following conditions. Set the temperature of the heated lid to 105°C.

	STEP	TEMPERATURE	TIME	NUMBER CYCLES
1	Initialization	98°C	45 seconds	1
2	Denaturation	98°C	15 seconds	12
	Annealing	60°C	30 seconds	
	Extension	72°C	30 seconds	
3	Final Extension	72°C	1 minute	1
4	Final Hold	4°C	HOLD	—

- 3.4.2** _____ Pulse-spin the Twist UDI Primer plate.
- 3.4.3** _____ Add 10 μl of Twist UDI Primer from the provided 96-well thermal cycling plate to each cDNA library reaction and mix well by gentle pipetting.
- 3.4.4** _____ Add 25 μl of KAPA HiFi HotStart ReadyMix to each cDNA library reaction and mix well by gentle pipetting. Close the strip tubes or seal the plate with adhesive PCR plate foil.
- 3.4.5** _____ Pulse-spin sample plate or tubes and immediately transfer to the thermal cycler. Start the program.

STAGE 5: PCR PRODUCT CLEAN UP

- 3.5.1** _____ While the cDNA library amplification is incubating, equilibrate the Twist DNA Purification Beads at room temperature and freshly prepare a solution of 500 μl of 80% ethanol per sample.
- 3.5.2** _____ When the cDNA library amplification incubation is finished, remove the reactions from the thermal cycler. Vortex the pre-equilibrated Twist DNA Purification Beads until mixed.
- 3.5.3** _____ Add 50 μl (1x) of homogenized Twist DNA Purification Beads to each cDNA library amplification reaction. Mix well by vortexing.
- 3.5.4** _____ Incubate the samples for 5 minutes at room temperature.
- 3.5.5** _____ Place the samples on a magnetic plate for 1 minute or until supernatant is clear.
- 3.5.6** _____ Without removing the plate or tubes from the magnetic plate, remove and discard the supernatant.
- 3.5.7** _____ Wash the bead pellet by gently adding 200 μl of 80% ethanol (do not disturb the pellet), incubate for 1 minute, then remove and discard the ethanol.
- 3.5.8** _____ Repeat this wash once, for a total of two washes, while keeping the samples on the magnetic plate.
- 3.5.9** _____ Carefully remove all remaining ethanol with a 10 μl pipette, making sure not to disturb the bead pellet.
- 3.5.10** _____ Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not over dry the bead pellet.
- 3.5.11** _____ Remove the plate or tubes from the magnetic plate and add 22 μl of nuclease-free water to each sample. Mix by pipetting until homogenized.
- 3.5.12** _____ Incubate at room temperature for 2 minutes.
- 3.5.13** _____ Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.

3.5.14 _____ Transfer 20 µl of the clear supernatant containing the amplified indexed libraries to a clean thin-walled PCR 0.2 ml strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

3.5.15 _____ Place samples on ice and proceed to QC the purified cDNA libraries by quantifying with Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay and assess library size with Agilent TapeStation D5000 Reagents and ScreenTape. Please see the “Quality Control Procedures” section for details.

THIS IS A SAFE STOPPING POINT.

STORE SAMPLES AT -20°C IF NOT IMMEDIATELY PROCEEDING TO MODULE 4.

REVOKED

MODULE 4 SARS-CoV-2 CAPTURE

STAGE 1: POOLING STRATEGY

- 4.1.1** On ice, thaw the Twist SARS-CoV-2 Research Panel, Twist Universal Blockers, Twist Blocker Solution, Twist Fast Hybridization Mix, Twist Hybridization Enhancer, and the amplified indexed libraries.
- 4.1.2** Calculate the input volume (in μl) of the library needed for hybridization by dividing 187.5 by the concentration (ng/ μl) of each amplified indexed library. Multiplexing eight libraries per hybridization reaction should have a total mass of 1,500 ng.
- 4.1.3** Transfer the calculated volumes from each amplified indexed library to a 0.2 ml thin-walled PCR strip tube. Each hybridization reaction should contain eight libraries per pool.

STAGE 2: TARGET ENRICHMENT (HYBRIDIZATION)

- 4.2.1** Mix each reagent by flicking the tube.
- 4.2.2** Add the following volumes of reagents to each capture pool to create a hybridization reaction. Mix by flicking.

REAGENT	VOLUME
Twist SARS-CoV-2 Research Panel	4 μl
Twist Universal Blockers	8 μl
Twist Blocker Solution	5 μl

- 4.2.3** Pulse-spin the tube and ensure there are minimal bubbles present. Dry the hybridization reaction completely using a SpeedVac system with low heat.
- 4.2.4** Heat the Twist Fast Hybridization Mix at 65°C for 10 minutes, or until all precipitate is dissolved. Vortex and use immediately. Do not allow the Twist Fast Hybridization Mix to cool to room temperature.
- 4.2.5** Program the 96-well thermal cycler with the following conditions. Set the heated lid to 85°C:

	TEMPERATURE	TIME
1	95°C	HOLD
2	95°C	5 minutes
3	60°C	HOLD

- 4.2.6** _____ Resuspend the dried hybridization reaction with 20 μ l of Twist Fast Hybridization Mix. Mix by gentle flicking.
- 4.2.7** _____ Pulse-spin the tube(s) and ensure there are minimal bubbles present.
- 4.2.8** _____ Add 30 μ l of Twist Hybridization Enhancer to the hybridization reaction. Mix by gentle flicking.
- 4.2.9** _____ Pulse-spin the tube(s) and ensure there are minimal bubbles present. Transfer the tube(s) to the preheated thermal cycler. Skip to the 5 minutes at 95°C step.
- 4.2.10** _____ Incubate the hybridization reaction at 60°C for 2 hours in a thermal cycler with the lid set to 85°C.

STAGE 3: STREPTAVIDIN BINDING OF TARGETS AND HYBRIDIZATION WASHES

- 4.3.1** _____ After 1 hour of incubation, equilibrate the Twist Fast Binding Buffer, Twist Streptavidin Binding Beads and Twist DNA Purification Beads to room temperature for 30 minutes. Visually check that there are no precipitates present.
- 4.3.2** _____ For each hybridization reaction, aliquot 450 μ l of Twist Fast Wash Buffer 1 and preheat to 68°C. For each hybridization reaction, aliquot 700 μ l of Twist Wash Buffer 2 and preheat to 48°C.
- 4.3.3** _____ On ice, thaw the KAPA HiFi HotStart ReadyMix and Twist Amplification Primers.
- 4.3.4** _____ Vortex the pre-equilibrated Twist Streptavidin Binding Beads until mixed.
- 4.3.5** _____ Add 100 μ l Twist Streptavidin Binding Beads to a 1.5 ml microcentrifuge tube. Prepare one tube for each hybridization reaction.
- 4.3.6** _____ Add 200 μ l of Twist Fast Binding Buffer to the tube(s) and mix by pipetting.
- 4.3.7** _____ Place the tube(s) on a magnetic stand for 1 minute, then remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand.
- 4.3.8** _____ Repeat the wash two more times, for a total of three washes.
- 4.3.9** _____ After removing the clear supernatant from the third wash, add a final 200 μ l of Twist Fast Binding Buffer and resuspend the beads by vortexing until homogenized.
- 4.3.10** _____ After the hybridization is complete, open the thermal cycler lid and, without removing the tubes from the block, quickly transfer the volume of each hybridization reaction (including the Twist Hybridization Enhancer) into a corresponding tube of washed streptavidin binding bead mix. Mix by pipetting and flicking.

- 4.3.11** _____ Mix each tube of streptavidin binding bead mix for 30 minutes at room temperature on a thermomixer set to a speed sufficient to keep the solution mixed. Do not vortex or mix aggressively.
- 4.3.12** _____ Remove the tube(s) containing the streptavidin binding bead mix from the mixer and pulse-spin to ensure all solutions are at the bottom of the tube(s).
- 4.3.13** _____ Place the tube(s) on a magnetic stand for 1 minute.
- 4.3.14** _____ Remove by pipette and discard the clear supernatant including the Twist Hybridization Enhancer. Do not disturb the bead pellet. A trace amount of Twist Hybridization Enhancer may be visible after supernatant removal and throughout each wash step. It will not affect the final capture product.
- 4.3.15** _____ Remove the tube(s) from the magnetic stand and add 200 μ l of preheated Twist Fast Wash Buffer 1. Mix by pipetting.
- 4.3.16** _____ Incubate the tube(s) for 5 minutes at 68°C.
- 4.3.17** _____ Place the tube(s) on a magnetic stand for 1 minute.
- 4.3.18** _____ Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.
- 4.3.19** _____ Remove the tube(s) from the magnetic stand and add an additional 200 μ l of preheated Twist Fast Wash Buffer 1. Mix by pipetting.
- 4.3.20** _____ Incubate the tube(s) for 5 minutes at 68°C.
- 4.3.21** _____ Pulse-spin to ensure all solutions are at the bottom of the tube(s).
- 4.3.22** _____ Transfer the entire volume (~200 μ l) into a new 1.5 ml microcentrifuge tube, one per hybridization reaction. Place the tube(s) on a magnetic stand for 1 minute.
- 4.3.23** _____ Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.
- 4.3.24** _____ Remove the tube(s) from the magnetic stand and add 200 μ l of preheated Twist Wash Buffer 2. Mix by pipetting, then pulse-spin to ensure all solutions are at the bottom of the tube(s).
- 4.3.25** _____ Incubate the tube(s) for 5 minutes at 48°C.
- 4.3.26** _____ Place the tube(s) on a magnetic stand for 1 minute.
- 4.3.27** _____ Remove by pipette and discard the clear supernatant. Make sure to not disturb the bead pellet.
- 4.3.28** _____ Repeat the wash two more times, for a total of three washes.

4.3.29 After the final wash, remove all traces of supernatant using a 10 µl pipette. Proceed immediately to the next step. Do not allow the beads to dry.

4.3.30 Remove the tube(s) from the magnetic stand and add 45 µl of nuclease-free water. Mix by pipetting until homogenized. Incubate this solution, hereafter referred to as the streptavidin binding bead slurry, on ice.

STAGE 4: POST CAPTURE PCR AMPLIFICATION

4.4.1 Program the thermal cycler with the following conditions. Set the heated lid to 105°C.

STEP	TEMPERATURE	TIME	NUMBER CYCLES
1	98°C	45 seconds	1
2	98°C	15 seconds	16
	60°C	30 seconds	
	72°C	30 seconds	
3	72°C	1 minute	1
4	4°C	HOLD	—

4.4.2 If the streptavidin binding bead slurry has settled, mix by pipetting.

4.4.3 Transfer 22.5 µl of the streptavidin binding bead slurry to a thin-walled PCR 0.2 ml strip-tube(s). Keep on ice until the next step. Store the remaining 22.5 µl streptavidin binding bead slurry at –20°C for future use.

4.4.4 Mix the KAPA HiFi HotStart ReadyMix and Twist Amplification Primers by vortexing for 2 seconds.

4.4.5 Prepare a PCR master mix by adding the following reagents to the tube(s) containing the streptavidin binding bead slurry. Mix by pipetting.

REAGENT	VOLUME PER REACTION
Streptavidin binding bead slurry	22.5 µl
Twist Amplification Primers, ILMN	2.5 µl
KAPA HiFi HotStart ReadyMix	25 µl
Total	50 µl

4.4.6 Pulse-spin the tubes, transfer them to the thermal cycler, and start the cycling program.

STAGE 5: POST CAPTURE LIBRARY CLEAN UP

- 4.5.1** While the streptavidin binding bead slurry is amplifying, equilibrate the Twist DNA Purification Beads at room temperature and prepare a fresh solution of 80% ethanol (500 μ l per sample).
- 4.5.2** When the thermal cycler program is complete, remove the reactions from the thermal cycler. Vortex the pre-equilibrated Twist DNA Purification Beads until mixed.
- 4.5.3** Add 90 μ l (1.8x) of homogenized Twist DNA Purification Beads to each reaction. Mix well by vortexing.
- 4.5.4** Incubate the samples for 5 minutes at room temperature.
- 4.5.5** Place the samples on a magnetic plate for 1 minute.
- 4.5.6** Without removing the plate, or tubes from the magnetic plate, remove and discard the supernatant.
- 4.5.7** Wash the bead pellet by gently adding 200 μ l of 80% ethanol (do not disturb the pellet), incubate for 1 minute, then remove and discard the ethanol.
- 4.5.8** Repeat this wash step twice while keeping the samples on the magnetic plate.
- 4.5.9** Carefully remove all remaining ethanol with a 10 μ l pipette. Do not disturb the bead pellet.
- 4.5.10** Air-dry the bead pellet on the magnetic plate for 5–10 minutes or until the bead pellet is dry. Do not over dry.
- 4.5.11** Remove the plate or tubes from the magnetic plate and add 32 μ l of nuclease-free water to each sample. Mix by pipetting until homogenized.
- 4.5.12** Incubate at room temperature for 2 minutes.
- 4.5.13** Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.
- 4.5.14** Transfer 30 μ l of the clear supernatant containing the final captured amplified sample to a clean thin-walled PCR 0.2 ml strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.
- 4.5.15** Place samples on ice and QC the final captured amplified samples by quantifying with Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay. Assess library size with Agilent TapeStation D1000 Reagents and ScreenTape. Please see the “Quality Control Procedures” section for details.

THIS IS A SAFE STOPPING POINT.

STORE SAMPLES AT -20°C IF NOT IMMEDIATELY PROCEEDING TO MODULE 5.

MODULE 5 ILLUMINA NEXTSEQ PREPARATION AND INSTRUMENTATION

STAGE 1: GENERATE SAMPLE SHEET AND LOAD EXPERIMENT ON THE SEQUENCER

- 5.1.1** Generate a sequencing sample sheet (.csv) containing the sample information and corresponding indexing barcodes. A template sample sheet, listed under the 'Template_Sample_Sheet' tab, can be found in the IFU Supplemental Information. Indexing barcode sequences can be found under the 'Barcodes' tab in the IFU Supplemental Information. This information can be found at twistbioscience.com/IFU.
- 5.1.2** After generating the sample sheet, open the Illumina Local Run Manager software and click '+Create Run', and then 'GenerateFASTQ'.
- 5.1.3** Click 'Import Sample Sheet' and make sure the following parameters are filled out correctly:
- Run name
 - Run description
 - Read type should be selected as 'Single Read'
 - Index reads should be selected as '2'.
 - Read lengths should be filled out as '148' for Read 1, '10' for Index 1, and '10' for Index 2.
 - Sample ID's with corresponding indexing barcodes.
- 5.1.4** Click 'Save Run'.

STAGE 2: DILUTE AND POOL FINAL CAPTURED AMPLIFIED SAMPLES

GUIDELINES

Prepare aliquots of 4 nM PhiX control as follows:

- Thaw a tube of 10 nM Illumina PhiX control stock (10 µl/tube) and Illumina Resuspension Buffer (RSB).
- Combine 15 µl of RSB and 10 µl of 10 nM Illumina PhiX control in a 1.5 ml microcentrifuge tube to obtain a concentration of 4 nM.
- Vortex briefly and then pulse-spin.
- Aliquot 5 µl of 4 nM PhiX control into a clean 1.5 ml microcentrifuge tube.
- Store aliquots at -20°C until ready for use.

- 5.2.1** If capture libraries or 4 nM PhiX control were frozen, thaw samples on ice.
- 5.2.2** Thaw the Illumina Reagent Cartridge and Illumina HT1 Hybridization Buffer for sequencer loading in a nuclease-free water bath at room temperature for 45 minutes.
- 5.2.3** Chill the Illumina HT1 Hybridization Buffer on ice after thawing occurs.
- 5.2.4** Equilibrate the Illumina NextSeq 550 Flow Cell Cartridge at room temperature for at least 30 minutes.

5.2.5 According to the concentration values and library size obtained on Step 4.5.15, calculate the molarity (nM) of your final captured amplified samples using the following formula:

$$\text{(Qubit concentration in ng/}\mu\text{l)} / (660 \times \text{Average library size}) \times 10^6$$

5.2.6 In a clean 1.5 ml microcentrifuge tube, dilute the final captured amplified samples to a 4 nM concentration with nuclease-free water.

5.2.7 Pool equal volumes of the diluted 4 nM final captured amplified samples into a clean 1.5 ml tube to generate the final captured amplified pool.

5.2.8 Place the final captured amplified pool on ice and proceed to perform your final QC by quantifying and validating the size of the final captured amplified pool using the Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay and Agilent TapeStation D1000 High Sensitivity DNA kit. Please see the “Quality Control Procedures” section for details.

STAGE 3: DILUTE AND DENATURE FINAL CAPTURED AMPLIFIED POOL

5.3.1 Freshly dilute stock concentration of 1 M Teknova NaOH to 0.2 M NaOH by adding 800 μl of water to 200 μl of 1 M Teknova NaOH into a clean 1.5 ml microcentrifuge tube.

5.3.2 To denature the final captured amplified pool, add 5 μl of the final captured amplified pool at 4 nM and 5 μl of the 0.2 M NaOH to a clean 1.5 ml microcentrifuge tube. Additionally, in a different 1.5 ml microcentrifuge tube, add 5 μl of the 0.2 M NaOH and 5 μl of the 4 nM PhiX control.

5.3.3 Briefly vortex and spin down each tube.

5.3.4 Incubate for 5 minutes at room temperature.

5.3.5 Add 5 μl of 200 mM Tris-HCl (pH 7) to the denatured final captured amplified pool and the denatured PhiX control.

5.3.6 Briefly vortex each tube and spin down.

5.3.7 Add 985 μl of the chilled Illumina HT1 Hybridization Buffer to each tube to get the denatured and diluted final captured amplified pool and PhiX control at 20 pM. Vortex and pulse-spin the tubes and ensure there are minimal bubbles present.

5.3.8 To further dilute the denatured PhiX control to 1.3 pM, in a clean 1.5 ml microcentrifuge tube, add 84.5 μl of 20 pM PhiX control to 1215.5 μl of pre-chilled Illumina HT1 Hybridization Buffer.

5.3.9 Vortex and spin down.

- 5.3.10** _____ To further dilute the final sequencing pool to 1.3 pM, in a clean 1.5 ml microcentrifuge tube, add 84.5 µl of 20 pM final captured amplified pool with 13 µl of 1.3 pM PhiX control and 1202.5 µl of pre-chilled Illumina HT1 Hybridization Buffer. This is your final loading library with 1% PhiX control spike-in.
- 5.3.11** _____ Vortex, spin down and place on ice until ready to load into the sequencer.
- 5.3.12** _____ Sequence libraries as soon as possible after pooling. Pooled libraries are stable for up to 7 days at -25°C to -15°C.

STAGE 4: LOADING THE SEQUENCER

- 5.4.1** _____ Unwrap the Illumina NextSeq 550 Flow Cell Cartridge to remove packaging, including the clear plastic wrapper.
- 5.4.2** _____ Clean the surface of the Illumina NextSeq 550 Flow Cell using a lint-free alcohol wipe. Dry the surface using a clean lint-free tissue.
- 5.4.3** _____ Click 'Experiment' on the sequencer Home Screen.
- 5.4.4** _____ On the Select Assay screen, click 'Sequence'.
- 5.4.5** _____ Setup the run by opening 'Local Run Manager' and clicking 'Base Space Sequence Hub' settings. Log into an Illumina BaseSpace account.
- 5.4.6** _____ Click 'Next' and select a run from the list of available runs that have previously been imported on the Illumina Local Run Manager software.
- 5.4.7** _____ Confirm run parameters.
- Run Name: Name of the run as assigned in Local Run Manager.
 - Library ID: Name of the pooled libraries as assigned in Local Run Manager.
 - Recipe: Name of the recipe, either NextSeq High or NextSeq Mid depending on the reagent cartridge used for the run.
 - Read Type: Single-Read
 - Read Length: 148 cycles for read 1, 10 for Index 1, 10 for Index 2.
- 5.4.8** _____ Click 'Next'.
- 5.4.9** _____ Place the Illumina NextSeq 550 Flow Cell in the appropriate orientation on the sequencer platform as seen on the sequencer Home Screen.
- 5.4.10** _____ Click "Load", and then 'Next'.
- 5.4.11** _____ Remove the spent Reagents Container and discard the contents in accordance with applicable standards.

- 5.4.12** Slide the empty Reagents Container into the buffer compartment until it stops. An audible click indicates that the container is in position.
- 5.4.13** Remove the used Buffer Cartridge from the upper compartment.
- 5.4.14** Slide a new Buffer Cartridge into the buffer compartment until it stops.
- 5.4.15** Close the buffer compartment door and click 'Next'.
- 5.4.16** Remove the used Reagent Cartridge from the reagent compartment. Dispose of unused contents in accordance with applicable standards.
- 5.4.17** Once the new Reagent Cartridge is thawed, mix the reagents by inverting the cartridge.
- 5.4.18** Gently tap the cartridge to remove any water.
- 5.4.19** Using a clean 1 ml pipette tip, pierce the seal in reservoir #10.
- 5.4.20** Add the entire 1.3 ml of the final sequencing pool with 1% PhiX control spike-in into reservoir #10.
- 5.4.21** Gently tap the Reagent Cartridge to assure the final loading aliquot is at the bottom of the reservoir.
- 5.4.22** Slide the Reagent Cartridge into the reagent compartment until the cartridge stops, and then close the reagent compartment door.
- 5.4.23** Click 'Load', and then 'Next'.
- 5.4.24** When the automated check is complete, click 'Start'. The sequencing run should begin.

STAGE 5: INSTRUMENT SPECIFICATION

If using the NextSeq 500/550 or NextSeq 550Dx, refer to the NextSeq 500 System Guide (document # 15046563), NextSeq 550 System Guide (document # 15069765), or NextSeq 550Dx Instrument Reference Guide (document # 1000000009513). If using the NextSeq 550Dx, use RUO mode.

DESCRIPTION OF BIOTIA USER PORTAL (DATA TRANSFER AND REPORTING)

Biotia COVID-DX (v1) software has been built to analyze the SARS-CoV-2 NGS-Assay and generate an FDA EUA clinical report. Users can register on the Biotia Portal, place an order, transfer the sequencing data (demultiplexed FASTQ files), and retrieve the reports.

Access the Biotia COVID-DX (v1) Software

After sequencing, users can upload their FASTQ files through our simple user interface (the Biotia Portal is linked via biotia.io). Users must obtain an EUA serial number prior to upload of FASTQs. After upload of FASTQs, users will receive the FDA EUA report, which describes presence/absence of the SARS-CoV-2 virus.

The SARS-CoV-2 NGS Assay includes access to bioinformatic analysis for 101 samples (per 96-sample kit). To receive your Biotia analysis credits, register on the Biotia Portal by going to biotia.io and enter your unique order number, which is emailed to you after purchasing the SARS-CoV-2 NGS Assay. Credits for Biotia COVID-DX (v1) expire one (1) year after purchasing the SARS-CoV-2 NGS Assay.

Biotia Portal Registration

- Visit biotia.io and select the 'Order Test' link. This will connect you to our Biotia Portal, where you can register for a new user account using a valid email address.
- Select the option 'Researcher'.
- Complete all fields, then select 'Register'.
- We will review your registration request and confirm your activation within one business day. Upon confirmation of activation, the system will send you a unique link to the portal via email that you can follow to set your password.
- Click the link provided in the email and create a unique password. (The password must contain: an uppercase character, a lowercase character, a minimum of 10 characters, and at least one number.)
- Once the registration is complete, you can log in as a 'Researcher' user with your email and password.

Biotia Portal Navigation and User Profile

- After logging in, you will enter the Landing Page of the Biotia Portal. You can review all of your previously ordered tests.
- By selecting 'Menu' on the left side, you can select from all available functions.
- By selecting the 'user' icon in the top-right corner, you can navigate to the User Profile page. There you can edit your profile information (i.e., modify name, add new email address, etc.).
- By selecting 'Sign out' in the top-right corner, you can sign out of the Biotia Portal.

Creating a New Order

- You can register a new order by selecting 'COVID-DX' in the Menu Bar.
- Choose the user email and institution address to be used for this order, accept Biotia Terms of Use, and agree to responsibility for payment of charges (when applicable).
- Provide your unique SARS-CoV-2 NGS Assay EUA serial number to access your credits for the Biotia COVID-DX (v1) software.
- Select 'Add Tests' and complete required Test Registration information.
- Upload the provided 'Sample Submission Form' to our secure cloud storage on Azure, including data type, sequence file names, and additional sequencing information.
- Please see instructions above for demultiplexing. Upload the 4 demultiplexed FASTQ files by either using the web interface or in a batch using the Azure secure CLI with a temporary credential issued by the Portal.
- If you want to save and return to the Order Registration later, select 'Save' in the top-right corner. You can retrieve and edit the saved order from the Home Screen Test List by selecting the pencil icon next to the order. The saved order will appear as Order Status: 'Under Registration'.
- Once the order is complete, select 'Submit'. You may use either of the 'Submit' buttons at the top or the bottom of the page. This will trigger the Biotia Portal to submit the order and set the status of the order to 'Registered'.
- The User is able to cancel the Order/Test by clicking the 'X' on the left in the order's row. Cancellation of order is only available before the sample begins to be processed.

Reporting

- Once the analyses are complete, a Biotia Test Report will be generated and made available to the user on the Biotia Portal. The diagnostic report states if SARS-CoV-2 was detected. If also prescribed, a variant report may be separately generated if the appropriate sequence quality controls pass. The variant report states the determined PANGO lineage for the detected SARS-CoV-2 and lists SARS-CoV-2 genetic mutations identified.
- Users will receive an email notifying them that their reports are ready for review and download.

DESCRIPTION OF COVID-DX (V1) SOFTWARE AND EUA REPORT

The pipeline software is contained within Docker images orchestrated by Cromwell / WDL backed by Azure Batch.

Pre-processing and Quality Control

- The program cut adapt trims adapter sequences using the standard Illumina adapter sequence “AGATCGGAAGAGC” and a minimum read length of 20 bp. Samples with higher than 50,000,000 reads are subsampled to 50,000,000 reads using seqtk before further analysis.
- FASTQs are split for parallelization and aligned against the genomes of SARS-CoV-2 virus (NC_045512.2), 26 other viral and bacterial respiratory pathogens, and the human genome using Bowtie2 run in local mode.
- The algorithm to detect SARS-CoV-2 uses only reads that map unambiguously to the SARS-CoV-2 reference (NC_045512.2) in order to minimize false positives due to cross-hybridization of the probes or cross-contamination during the Twist NGS hybrid capture. Unambiguously mapped reads are extracted using samtools-view.
- Duplicates and read statistics are calculated with Picard (2.23.0) and the CollectHSMetrics function, for which the source code is freely available at github.com/broadinstitute/picard/releases/tag/2.23.0.
- The depth of reads at each site across the SARS-CoV-2 genome is calculated using samtools-depth.
- Test samples and/or positive controls with fewer than 10,000 total reads are assigned as invalid.

SARS-CoV-2 Presence/Absence Calculation

- Detection of the SARS-CoV-2 virus is based on the degree to which the genome was recovered in sequencing. The more genome that is recovered, the more likely it is that the virus is present.
- A sample-specific integral is computed by calculating the coverage at 1x depth using a sliding window scheme (with a window size of 1,000 and step size of 100). This metric is then log transformed. A threshold of 8.6 for samples with less than or equal to 10,000 bases on target or 9.6 for samples with more than 10,000 bases on target determines if SARS-CoV-2 is present or absent. The integral is calculated using the R statistical software package (v4.0.1).

SARS-CoV-2 Mutations and Lineage Detection

- PANGO lineage is determined using Pangolin (minimum v4.0.3) via PangoleARN (minimum version 04/15/2022). Genetic mutations are determined by aligning the test strain to the SARS-CoV-2 reference genome (NC_045512.2) with information on gene location/site and alteration. An overall 90% genome coverage is necessary to confidently call lineages (manual review will confirm sufficient coverage). If the overall coverage is less than 90%, neither the lineage nor mutation calls will be reported. A minimum of 10 reads/mutation is required to call mutations.

Report Generation

- Outputs are consolidated in a summary JSON, which is processed by an Azure Function to generate a final report for distribution to the requester. The diagnostic report states if SARS-CoV-2 was detected. If also prescribed, a variant report may be separately generated if the appropriate sequence quality controls pass. The variant report states the determined PANGO lineage for the detected SARS-CoV-2 and lists SARS-CoV-2 genetic mutations identified.

CONTROLS

The following controls are used during the protocol:

DESCRIPTION OF CONTROL	TYPE OF CONTROL	CONTROL TO SAMPLE RATIO	AMOUNT USED	USED TO MONITOR	EXPECTED RESULTS	VENDOR AND PART NUMBER
Viral transport media (VTM)	Negative/ no template control (NTC)	One control for 96 test samples	250 µl	VTM is neat and does not contain human DNA or human cellular material. NTC is needed to eliminate the possibility of sample contamination on the assay run and is used with each extraction batch through sequencing.	Negative for SARS-CoV-2 targets detected	N/A
VERO E6 cells [ATCC® CRL-1586] spiked into VTM	Negative extraction control (NEC)	One control for 96 test samples	~4.0–4.5 x 10 ⁴ total cells (20 µl) spiked into 230 µl VTM	NEC serves as both a negative extraction control to monitor for any cross-contamination that occurs during the extraction process, as well as an extraction control to validate extraction reagents and successful RNA extraction. This NEC gives a detectable result throughout the workflow (QC steps); however it yields off-target reads that are removed bioinformatically and detected as negative for SARS-CoV-2.	Negative for SARS-CoV-2 targets detected	ATCC® CRL-1586
Positive Twist RNA template control - Synthetic SARS-CoV-2 RNA Control 2 (MN908947.3) in Agilent Gene Expression Universal Reference RNA (Human)	Positive control (PC)	One control for 96 test samples	2,560 copies/ml (3.2x LoD)	PC is needed to verify that the assay run is performing as intended. The positive control is made of six RNA fragments that are 5,000 bp in length spanning the entire SARS-CoV-2 genome (MN908947.3). All coding and non-coding regions of the viral genome are included in these controls, except for the polyadenylated region. Each RNA fragment is made by <i>in vitro</i> transcription using the DNA template as reference (see detailed description below).	Positive for SARS-CoV-2 targets detected	Twist: Comes with the SARS-CoV-2 NGS Assay kit Agilent: 740000-41
Agilent Gene Expression Universal Reference RNA (Human)	Internal control (IC)	One control for 96 test samples	3.85 ng/µl, 50 ng total	IC is used to validate the SARS-CoV-2 reagents and successful library generation, hybridization, and post hybridization PCR.	Negative for SARS-CoV-2 targets	Agilent: 740000-41

Positive RNA Control Generation

The positive control used in this assay is made by *in vitro* transcription from synthesized DNA generated by a silicon-based writing platform. DNA pieces 1,800 bp in length were written and assembled into larger fragments. Each fragment is approximately 5,000 bp in length spanning the entire SARS-CoV-2 virus (MN908947.3). All coding and non-coding regions of the viral genome are included in these controls, except for the polyadenylated region of the virus.

INTERPRETATION OF RESULTS

All test controls should be examined prior to interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted.

1. SARS-CoV-2 NGS Assay Controls – No Template, Negative Extraction, Positive, and Internal

All positive and negative controls will be assessed for presence/absence of SARS-CoV-2 with the same model as clinical samples with the same threshold applied as for clinical samples. Additionally, the number of reads mapped to the human genome will provide an internal control in each individual sample to be called valid. Calculation of the validity of the sample: if the sample is a test sample or positive control, it must have at least 10,000 reads post minimum read length filtering and at least 10,000 on-target bases within GrC38 to be deemed valid. The minimum number of reads does not apply to negative controls to validate.

The metric used to call the presence/absence of SARS-CoV-2 computes the integral under a curve created by calculating the coverage at 1x depth using a sliding window scheme (with a window size of 1,000 and step size of 100). This metric was then log transformed. We have set a threshold of 8.6 if samples had less than 10,000 bases on target or 9.5 if samples had more than 10,000 bases on target. If the VTM (NTC) is positive it requires rerunning the samples for ruling out possible cross-contamination.

If any control does not perform as described above and in the table below, run is considered invalid and all specimens are repeated from the extraction step.

CONTROL	EXPECTED RESULT TO PASS
Negative/No template control (VTM)	Valid if 1) SARS-CoV-2 is not detected (output metric < 8.6)
Negative extraction control (NEC)	Valid if 1) SARS-CoV-2 is not detected (output metric < 8.6)
Positive Twist RNA template control	Valid if 1) SARS-CoV-2 is detected (output metric \geq 8.6 if < 10,000 bp on SARS-CoV-2 target; output metric \geq 9.5 if > 10,000 bp on SARS-CoV-2 target) and 2) at least 10,000 reads post minimum read length filtering and at least 10,000 bp on-target bases within GrC38
Internal control (Gene Expression Universal Reference RNA (Human))	Valid if 1) SARS-CoV-2 is not detected (output metric < 8.6)
TEST SAMPLE	Valid if 1) at least 10,000 reads post minimum read length filtering and at least 10,000 on-target bases within GrC38

2. Examination and Interpretation of Patient Specimen Results

Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid and acceptable. If the controls are not valid, the patient results cannot be interpreted.

INTERPRETATION OF DIAGNOSTIC RESULT								
SARS-COV-2 IN TEST SAMPLE	CONTROLS				CONTROL RESULT	SAMPLE RESULT	INTERPRETATION	ACTION
	NTC	NEC	PC	IC				
<i>[Detected (+)/ Not Detected (-)]</i>	<i>[Passed/Failed]</i>				<i>[Passed/ Invalid]</i>			
Detected (+)	Passed	Passed	Passed	Passed	Passed	SARS-CoV-2 Detected	Positive for SARS-CoV-2 for the Test Sample.	Report results to physician, patient, and appropriate public health authorities.
Not Detected (-)	Passed	Passed	Passed	Passed	Passed	SARS-CoV-2 Not Detected	Negative for SARS-CoV-2 for the Test Sample.	Report results to physician, patient, and appropriate public health authorities.
Detected or Not Detected (+/-)	Failed	Passed or Failed	Passed or Failed	Passed or Failed	Invalid	Invalid	Invalid Test Sample result.	Quality control for the Test Sample has failed. Re-extract Test Sample and repeat SARS-CoV-2 NGS Assay.
Detected or Not Detected (+/-)	Passed or Failed	Failed	Passed or Failed	Passed or Failed	Invalid	Invalid	Invalid Test Sample result.	Quality control for the Test Sample has failed. Re-extract Test Sample and repeat SARS-CoV-2 NGS Assay.
Detected or Not Detected (+/-)	Passed or Failed	Passed or Failed	Failed	Passed or Failed	Invalid	Invalid	Invalid Test Sample result.	Quality control for the Test Sample has failed. Re-extract Test Sample and repeat SARS-CoV-2 NGS Assay.
Detected or Not Detected (+/-)	Passed or Failed	Passed or Failed	Passed or Failed	Failed	Invalid	Invalid	Invalid Test Sample result.	Quality control for the Test Sample has failed. Re-extract Test Sample and repeat SARS-CoV-2 NGS Assay.

For SARS-CoV-2 mutations and lineage calling, all controls must pass, and a detectable level of SARS-CoV-2 is needed. Specifically, lineage determination is only performed with an overall >90% genome coverage. Notably, if this criterion is not met, neither lineage determination nor mutation calling will be performed. Furthermore, a minimum of 10 reads per mutation is needed for a mutation at a given genomic position to be called (see Interpretation of PANGO Lineage Calling and Mutation Calling Results table below). The variant report includes the PANGO lineage, PangoLEARN version date, status of Quality Controls (pass/fail), and provides a list of detected genetic mutations compared to the reference (NC_045512.2). If the controls are not valid or SARS-CoV-2 was not detected, the patient results cannot be interpreted, and a variant report will not be generated.

INTERPRETATION OF PANGO LINEAGE CALLING AND MUTATION CALLING RESULTS				
SARS-COV-2 DIAGNOSTIC RESULT	QC FOR PANGO LINEAGE CALLING [PREREQUISITE FOR MUTATION CALLING]	QC FOR MUTATION CALLING AT A GENOMIC POSITION	RESULT	INTERPRETATION
	>90% OVERALL GENOMIC COVERAGE	POSITION COVERED BY >10 READS		
[Detected (+) / Not Detected (-) / Invalid]	[Passed / Failed / Not eligible]	[Passed / Failed / Not eligible]		
Detected (+)	Passed	Passed	PANGO lineage assignment AND A difference from the reference sequence (i.e. mutation) for the given genomic position was observed. This mutation is provided in a table in the variant report.	This test sample is positive for SARS-CoV-2. The PANGO lineage assignment for this positive test sample was determined. Mutation calling was performed at the given genomic position. The dominant nucleotide at this position differs from the reference sequence (NC_045512.2), and therefore a mutation is observed.
Detected (+)	Passed	Passed	PANGO lineage assignment AND No difference from the reference sequence (i.e. mutation) for the given genomic position was observed. No result is provided for the given genomic position in the variant report.	This test sample is positive for SARS-CoV-2. The PANGO lineage assignment for this positive test sample was determined. Mutation calling was performed at the given genomic position. However, the dominant nucleotide at this position is identical to the reference sequence (NC_045512.2), and therefore no mutation is observed.
Detected (+)	Passed	Failed	PANGO lineage assignment AND No result is provided for the given genomic position in the variant report.	This test sample is positive for SARS-CoV-2. The PANGO lineage assignment for this positive test sample was determined. Mutation calling cannot be performed due to insufficient quality controls.
Detected (+)	Failed	Not eligible for mutation calling	SARS-CoV-2 Mutation and Lineage Calling Invalid	This test sample is positive for SARS-CoV-2. Lineage and mutation calling cannot be performed due to insufficient quality controls.
Not Detected (-)	Not eligible for PANGO lineage calling	Not eligible for mutation calling	N/A	N/A
Invalid	Not eligible for PANGO lineage calling	Not eligible for mutation calling	N/A	N/A

The Biotia COVID-DX (v1) software will provide a clinical report including the presence/absence of the SARS- CoV-2 virus in a diagnostic report and, if also prescribed, its genetic mutations and PANGO lineage assignment in a separate variant report.

QUALITY CONTROL PROCEDURES

Quality Control steps follow nucleic acid extraction (Step 1.19), cDNA synthesis (Step 2.3.16), amplified indexed library generation (Step 3.5.15), the enrichment of the amplified samples (Step 4.5.15), and the dilution of the captured amplified pool (Step 5.2.8). These steps include analyses to determine fragment size and nucleic acid concentration.

STEP 1.1.9

The nucleic acid concentration values after nucleic acid extraction should be taken using the Thermo Fisher Scientific Qubit RNA High Sensitivity Quantitation Assay. The average value is 20 ng/μl, but can range from 2 to 45 ng/μl, depending on the integrity of each individual sample.

STEP 2.3.16

The nucleic acid concentration values after cDNA synthesis should be taken using the Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay. The average is 8 ng/μl, but could range from 1 to 12 ng/μl. Concentrations are dependent on the integrity and purity of starting RNA.

STEP 3.5.15

The nucleic acid concentration values after the amplified indexed library generation should be taken using the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantification Assay. The average value is 80 ng/μl, but could range from 12 to 150 ng/μl. Concentrations are dependent on the integrity and purity of starting cDNA and the number of cycles used in the library amplification step. Fragment size should be analyzed using Agilent TapeStation D5000 reagents and the ScreenTape platform. The average size of library fragments is 295 bp, but could range from 250 to 400 bp (Figure 2). Bimodal peaks are sometimes present during this step. This is often due to heteroduplex formations, which will not impact hybrid capture or NGS chemistry.

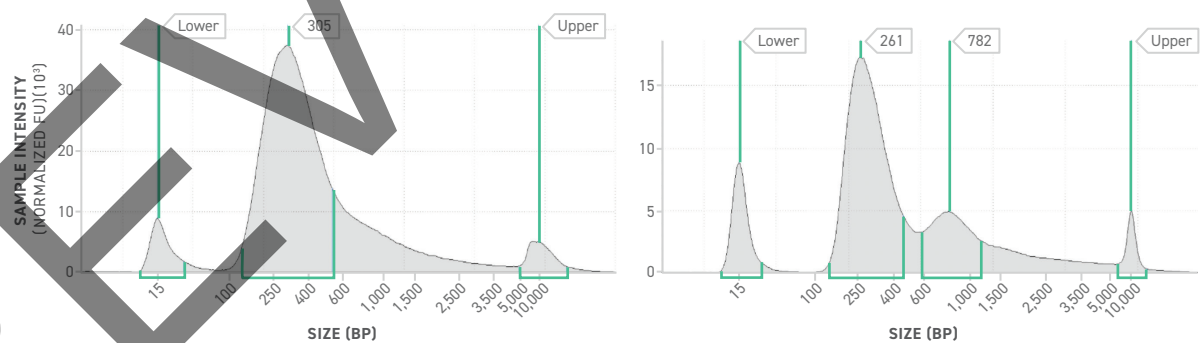


Figure 2: Amplified indexed library QC protocol examples with a single prominent and bimodal peaks. These electropherograms were generated by an Agilent TapeStation 5000 DNA analysis of cDNA library samples that were prepared as described. Note: you may observe a single prominent peak, or a bimodal peak, depending on the sample.

STEP 4.5.15

The nucleic acid concentration values for the final captured amplified sample should be taken using the Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantification Assay. This value is dependent on the viral titer of samples and viral detection. Fragment size should be analyzed using an Agilent Tapestation D1000 reagents and ScreenTape platform. Captured fragments should range between 300 to 425 bp (Figure 3).

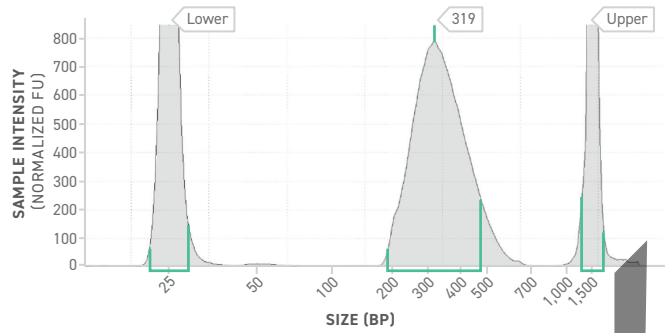


Figure 3: Individual capture QC protocol example with single peak. Electropherogram generated by an Agilent Tapestation D1000 DNA analysis of the enriched SARS-CoV-2 library samples that were prepared as described. Note the single prominent peak.

STEP 5.2.8

A final QC step is recommended to assess whether the correct library input is being loaded into the sequencer after the dilution of the captured amplified pool. The final nucleic acid concentration values for the captured amplified pool should be taken using the Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantification Assay. Fragment size should be analyzed using an Agilent Tapestation D1000 High Sensitivity reagents and ScreenTape platform. Captured fragments should range from 300 to 425 bp (Figure 4).

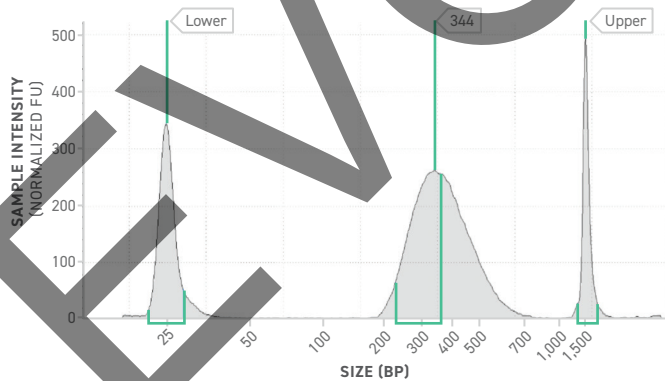


Figure 4: Additional pooled capture QC protocol example with single peak. Electropherogram generated by an Agilent Tapestation D1000 DNA analysis of the enriched SARS-CoV-2 final pooled library samples that were prepared as described. Note the single prominent peak.

Details on these protocols are specified below:

Thermo Fisher Scientific Qubit RNA High Sensitivity Quantification Assay Protocol

https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FQubit_RNA_Assay_QR.pdf&title=UXViaXQgUk5BIEFzc2F5IEtpdCBRdWljayBSZWZlcmVuY2U=

Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantification Assay Protocol

https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FQubit_dsDNA_HS_Assay_UG.pdf&title=VXNlciBHdWlkZTogUXViaXQgZHNETkEgSFMgQXNzYXkgS2l0cw==

Thermo Fisher Scientific Qubit dsDNA Broad Range Quantification Assay Protocol

https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FQubit_dsDNA_BR_Assay_UG.pdf&title=VXNlciBHdWlkZTogUXViaXQgZHNETkEgQllgQXNzYXkgS2l0cw==

Agilent TapeStation D5000 Protocol

https://www.agilent.com/cs/library/usermanuals/public/ScreenTape_D5000_QG.pdf

Agilent TapeStation D1000 Protocol

https://www.agilent.com/cs/library/usermanuals/public/ScreenTape_D1000_QG.pdf

Agilent TapeStation D1000 High Sensitivity Protocol

https://www.agilent.com/cs/library/usermanuals/Public/ScreenTape_HSD1000_QG.pdf

LIMITATIONS OF THE PROCEDURE

- Optimal performance of this test requires appropriate specimen collection, storage, and transport to the test site. Detection of SARS-CoV-2 virus may be affected by sample collection methods, patient factors (e.g., presence of symptoms), and/or stage of infection.
- The performance of this test was assessed using nasopharyngeal swab specimens in viral transport medium.
- False-negative results may arise from degradation of the virus RNA during shipping and storage.
- The impacts of vaccines, antiviral therapeutics, antibiotics, chemotherapeutic or immunosuppressant drugs have not yet been evaluated.
- Results should be interpreted by a trained professional in conjunction with the patient's history, clinical signs and symptoms, and epidemiological risk factors.
- Negative results do not preclude infection with the SARS-CoV-2 virus and should not be the sole basis for patient treatment and management, or public health decisions. Follow up testing should be performed according to the current CDC recommendations.
- Follow all safety laboratory precautions set by your institution guidelines. All specimens and samples should be handled as if they are infectious, using appropriate laboratory procedures. Personal protective equipment, such as gloves, eye protection, and lab coats, should be used when handling kit reagents and while performing this assay.
- Properly dispose of unused kit reagents and human specimens according to your institution's regulations.
- No modifications to this protocol are permitted under the EUA.
- The clinical performance has not been established in all circulating variants but is anticipated to be reflective of the prevalent variants in circulation at the time and location of the clinical evaluation. Performance at the time of testing may vary depending on the variants circulating, including newly emerging strains of SARS-CoV-2 and their prevalence, which change over time.
- The performance of this test was established based on the evaluation of a limited number of clinical specimens. The clinical performance has not been established in all circulating variants but is anticipated to be reflective of the prevalent variants in circulation at the time and location of the clinical evaluation. Performance at the time of testing may vary depending on the variants circulating, including newly emerging strains of SARS-CoV-2 and their prevalence, which change over time.
- Use of Twist Bioscience's SARS-CoV-2 NGS Assay is limited to laboratory personnel specifically instructed and trained in the operation of the Illumina NextSeq 500, NextSeq 550, and NextSeq 550Dx Sequencing System instrumentation and Next Generation Sequencing workflows as well as *in vitro* diagnostic procedures.
- Mutations may not be reported due to either failed QC at a certain genomic position, or because no mutation exists. Inferences should not be made regarding sites in the SARS-CoV-2 genome where mutation calling information was not provided. Lack of detection of a mutation does not preclude the possibility of a genetic mutation.
- The performance of mutation detection and identification was established based on the evaluation of a limited set of mutations. Performance for other mutations has not been evaluated by wet testing.
- Detection of a genomic mutation may not correlate with phenotypic gene expression.
- The performance of mutation/lineage calling when testing clinical samples with containing multiple SARS-CoV-2 strains was not evaluated.

- The clinical implications of lineage and mutation results are expected to evolve over time. Healthcare providers (HCPs) should check current guidance (e.g., COVID-19 Treatment Guidelines) to aid with interpretation of the results and seek additional up-to-date scientific information as needed. Patients should discuss their test results with the ordering HCP.
- PANGO lineage and mutation calling results from this test should not be used as the sole basis for patient management decisions.
- Time-sensitive treatment for COVID-19 should not be delayed while awaiting results of this test.
- This test was not specifically evaluated for use in conjunction with any specific mAb or antiviral product.
- Performance has only been established with samples positive by Twist Bioscience's EUA authorized SARS-CoV-2 NGS Assay. Positive specimens from other assays have not been evaluated and should not be used with this test.
- Failed (or invalid) results do not preclude presence of a specific SARS-CoV-2 PANGO lineage and should not be used as the sole basis for patient management decisions.
- Even if QC criteria are met, failed (or invalid) lineage results can occur for samples due to novel or missing lineages in the reference database.
- The Twist SARS-CoV-2 NGS Assay may fail to report PANGO lineage and mutations for SARS-CoV-2 positive samples identified using the test, especially in samples with lower SARS-CoV-2 levels that are around the limit of detection of the test for mutation and PANGO lineage reporting.
- The lineage determination result is specific to the version of Pangolin software used at time of testing and may vary when Pangolin software is updated.

CONDITIONS OF AUTHORIZATION FOR THE LABORATORY

Testing is limited to *laboratories - certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests in U.S. laboratories.* The SARS-CoV-2 NGS Assay is intended for use by qualified and trained clinical laboratory personnel specifically trained in NGS, PCR techniques, and *in vitro* diagnostic procedures. Personnel must use appropriate laboratory and personal protective equipment when handling the SARS-CoV-2 NGS Assay kits.

The SARS-CoV-2 NGS Assay Letter of Authorization, along with the authorized Fact Sheet for Healthcare Providers, the authorized Fact Sheet for Patients, and authorized labeling are available on the FDA website: <https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/vitro-diagnostics-euas>. However, to assist clinical laboratories using the SARS-CoV-2 NGS Assay, the relevant Conditions of Authorization are listed below:

- Authorized laboratories* using the SARS-CoV-2 NGS Assay must include with test result reports, all authorized Fact Sheets. Under exigent circumstances, other appropriate methods for disseminating these Fact Sheets may be used, which may include mass media.
- Authorized laboratories* using the SARS-CoV-2 NGS Assay must use the SARS-CoV-2 NGS Assay as outlined in the authorized labeling. Deviations from the authorized procedures, including authorized instruments, authorized extraction methods, authorized clinical specimen types, authorized control materials, authorized other ancillary reagents, and authorized materials required to use SARS-CoV-2 NGS Assay are not permitted.
- Authorized laboratories* that receive the SARS-CoV-2 NGS Assay must notify the relevant public health authorities of their intent to run the assay prior to initiating testing.
- Authorized laboratories* using the SARS-CoV-2 NGS Assay must have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.
- Authorized laboratories* must collect information on the performance of the SARS-CoV-2 NGS Assay and report to DMD/OHT7-OIR/OPEQ/CDRH (via email: CDRH-EUA-Reporting@fda.hhs.gov) and Twist/Biotia Tech Support (via email: customersupport@twistbioscience.com) any suspected occurrence of false positive or false negative results and significant deviations from the established performance characteristics of the SARS-CoV-2 NGS Assay of which they become aware.
- All laboratory personnel using your product must be appropriately trained in NGS, PCR techniques, *in vitro* diagnostic procedures and use appropriate laboratory and personal protective equipment when handling this kit, and use your product in accordance with the authorized labeling.
- Twist Bioscience, authorized distributors, and authorized laboratories using the SARS-CoV-2 NGS Assay must ensure that any records associated with this EUA are maintained until otherwise notified by the FDA. Such records will be made available to the FDA for inspection upon request.

*The letter of authorization refers to "Laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet requirements to perform high complexity tests" as "authorized laboratories."

PERFORMANCE CHARACTERISTICS

The following data outlined in the performance characteristics section were generated using the SARS-CoV-2 NGS Assay protocols and materials outlined in the Instructions for Use using nasopharyngeal (NP) swab samples. All sequencing data for this section were generated on an Illumina NextSeq 550. Data analysis was performed using the Biotia COVID-DX (v1) software, which is included in the SARS-CoV-2 NGS Assay kit.

Analytical Sensitivity

The analytical sensitivity (Limit of Detection [LoD]) study established the lowest concentration of SARS-CoV-2 (genome copies (cp)/ml) that can be detected by the SARS-CoV-2 NGS Assay at least 95% of the time. The preliminary LoD was established by testing 10-fold dilutions of the Twist Synthetic SARS-CoV-2 RNA Control 2 (MN908947.3, Catalog Number 102024). The preliminary LoD was confirmed by testing triplicates of 2-fold dilutions (2,560 copies/ml, 1,280 copies/ml, 640 copies/ml, 320 copies/ml, 160 copies/ml, 80 copies/ml, and 40 copies/ml) by spiking the quantified heat-inactivated SARS-CoV-2 (ATCC, VR-1986HK) into negative respiratory clinical matrices (NP swabs -clinical samples previously tested negative for SARS-CoV-2). The LoD was determined to be 800 copies/ml. The LoD (800 copies/ml) was replicated 30 times. 29/30 (96.67%) samples were positive.

Inclusivity

To evaluate the inclusivity of the assay, we processed six synthetic samples created by Twist Bioscience with known genetic sequences, including the original Wuhan coronavirus strain (MN908947), through laboratory processing and the bioinformatics pipeline. All six synthetic controls in the six samples were successfully called by the pipeline. The following information can be used to determine what strains and catalog numbers were synthesized in order to conduct this study:

- Twist Synthetic SARS-CoV-2 RNA Control 1 (MT007544.1), Catalog Number 102019
- Twist Synthetic SARS-CoV-2 RNA Control 2 (MN908947.3), Catalog Number 102024
- Twist Synthetic SARS-CoV-2 RNA Control 3 (LC528232.1), Catalog Number 102860
- Twist Synthetic SARS-CoV-2 RNA Control 4 (MT106054.1), Catalog Number 102862
- Twist Synthetic SARS-CoV-2 RNA Control 5 (MT188340), Catalog Number 102917
- Twist Synthetic SARS-CoV-2 RNA Control 6 (MT118835), Catalog Number 102918

We performed the following *in silico* inclusivity analysis. First, we downloaded 151,323 high-quality SARS-CoV-2 genome nucleotide sequences from GISAID (accessed 03/29/2021) in FASTA format. To enable efficient computational evaluation of these data, we indexed the combined FASTAs and split them into subsets for parallelization. We performed BLASTn alignments using 994 SARS-CoV-2 probes, with the PolyA tail removed, against the parallelized databases. We similarly eliminate the PolyA tail from alignment in our pipeline. We chose to present descriptive statistics of the data due to the breadth of high identity matches to SARS-CoV-2 genomes. Our analysis identified 16,503 unique sequences in the GISAID database with high identity matches. Percent identity is defined as the percent of nucleotides in the high scoring pair (the matching section of DNA) that match the query sequence (the probe). We identified 6,200 sequences with 100% mean identity (0 mean mismatches) to our probes; 16,503 sequences with $\geq 80\%$ mean percent identity to our probes, and 0 sequences with less than 80% mean identity to our probes. Given the high identity for the majority of probes in the majority of sequences, we expect to have a high probability of identifying the presence or absence of most known strains of SARS-CoV-2.

Our analysis identified 151,323 unique sequences in the GISAID database with high identity matches. Percent identity is defined as the percent of nucleotides in the high scoring pair (the matching section of DNA) that match the query sequence (the probe). We identified 50 sequences with 100% mean identity (0 mean mismatches) to our probes; 151,323 sequences with $\geq 80\%$ mean percent identity to our probes, and 0 sequences with less than 80% mean identity to our probes. Given the high identity for the majority of probes in the majority of sequences, we expect to have a high probability of identifying the presence or absence of most known strains of SARS-CoV-2. Homology analysis for $>150,000$ SARS-CoV-2 sequences downloaded from GISAID is shown in the following table.

HOMOLOGY ANALYSIS FOR $>150,000$ SARS-COV-2 SEQUENCES DOWNLOADED FROM GISAID

	MEAN	MEDIAN	5TH PERCENTILE	95TH PERCENTILE
Percent of Probes with $>80\%$ Homology	99.88%	100%	99.30%	100%
Percent of Probes with 100% Homology	92.62%	92.35%	88.23%	97.18%

Cross-Reactivity

To account for potential cross-reactivity of the SARS-CoV-2 NGS hybridization probes, we aligned probe sequences to 30 microbial genomes and the human genome along with the SARS-CoV-2 genome (31 total sequences).

	COMBINED GENOME
Date	2/16/21
Total Sequences Reviewed	31
Unique Sequences Reviewed	31
Sequences with 100% Mean Percent Identity	0
Sequences with $\geq 80\%$ Mean Percent Identity	2 NC_006577.2 (human coronavirus HKU1); 84% NC_004718 (SARS-CoV-1); $>80\%$
Sequences with $< 80\%$ Mean Percent Identity	29
Sequences with 0% Mean Percent Identity	29

ORGANISMS EXAMINED	ACCESSION	NUMBER OF PROBES WITH HOMOLOGY ABOVE 80%
Adenovirus (e.g. C1 Ad. 71)	NC_001405.1	0
Human Metapneumovirus (hMPV)	NC_039199.1	0
Parainfluenza virus 1	JQ901971.1	0
Parainfluenza virus 2	NC_003443.1	0
Parainfluenza virus 3	NC_001796.2	0
Parainfluenza virus 4	KF483663.1	0
Influenza A	AB284320.1	0
Influenza B	NC_002208.1	0
Enterovirus (e.g. EV68)	AY426531.1	0
Respiratory syncytial virus	NC_038235.1	0
Rhinovirus	ENA L24917 L24917.1	0
Haemophilus influenzae	Haemophilus_influenzae_ATCC_51907	0
Legionella pneumophila	Legionella_pneumophila_subsp_pneumophila_ATCC_33152	0
Mycobacterium tuberculosis	NC_000962.3	0
Streptococcus pneumoniae	Streptococcus_pneumoniae_ATCC_700669	0
Streptococcus pyogenes	Streptococcus_pyogenes_ATCC_12344	0
Mycoplasma pneumoniae	NZ_CP010546.1	0
Candida albicans	CP017623.1, CP017624.1, CP017625.1, CP017626.1, CP017627.1, CP017628.1, CP017629.1, CP017630.1	0
Pseudomonas aeruginosa	Pseudomonas_aeruginosa_ATCC_9027	0
Staphylococcus epidermidis	Staphylococcus_epidermidis_ATCC_12228	0
Streptococcus salivarius	Streptococcus_salivarius_ATCC_9759	0
Human coronavirus 229E	NC_002645.1	0
Human coronavirus OC43	NC_006213.1	0
Human coronavirus HKU1	NC_006577.2	3
Human coronavirus NL63	DQ445911.1	0
MERS-coronavirus	KT006149.2	0
Chlamydia pneumonia	NC_005043.1	0
Pneumocystis jirovecii (PCP)	GCA_001477535.1	0
Bordetella pertussis	CP011448.1	0
Human SARS-CoV-1	NC_004718	174
Human Genome	GrC38	0

For the analysis, 30 non-SARS-CoV-2 consensus genomes and the human genome were downloaded from NCBI as the negative sample cohort. These genomes are the same as those previously described for alignment. A BLASTn (NCBI) analysis was then performed to quantify the number of probes that had more than 80% homology with each of the genomes in the cohort. All organisms were determined to have no cross-reactivity with the probes used, except human coronavirus HKU1 with 3 out of 994 probes with 84.6% homology and SARS-CoV-1 with 174 out of 994 probes with > 80% homology. These cross-reactive probes are not expected to have significant clinical impact due to low probe coverage and/or currently low infection rates by these organisms. Reads that are aligned outside of the SARS-CoV-2 genome are rejected from presence / absence evaluation.

Clinical Evaluation

Clinical performance of the SARS-CoV-2 NGS Assay was evaluated by comparing results to an RT-PCR assay authorized by the FDA for use under Emergency Use Authorization. 60 clinical nasopharyngeal swab (NP) specimens were tested, including 30 SARS-CoV-2-positive and 30 SARS-CoV-2-negative specimens.

The positive and negative percent agreement was calculated in relation to the EUA RT-PCR comparator method and indicated in the following table.

		EUA RT-PCR COMPARATOR ASSAY		
		POSITIVE	NEGATIVE	% AGREEMENT (95% CI)
SARS-COV-2 NGS ASSAY	POSITIVE	29	0	PPA: 96.7% (83.3–99.4%)
	NEGATIVE	1	29	NPA: 100% (88.3–100%)
	INVALID	0	1	–

*One PCR negative sample did not yield sufficient reads using the SARS-CoV-2 NGS Assay to be called negative and labelled as INVALID.

Validation of SARS-CoV-2 Mutation Calling

A preliminary estimated LoD for detection of SARS-COV-2 mutations was determined based on testing a limited number of replicates of contrived samples composed of an RNA synthetic control (Alpha lineage, B.1.1.7, EPI_ISL_601443, PN103909, Twist Bioscience) with a known set of 32 mutations as compared to the reference SARS-CoV-2 (NC_045512.2) over the same regions, spiked into negative respiratory clinical matrices (NP swabs -clinical samples previously tested negative for SARS- CoV-2). This synthetic alpha control was spiked (in duplicate) to yield the final following concentrations: 800 copies/ml, 4,000 copies/ml, 16,000 copies/ml, 32,000 copies/ml, and 48,000 copies/ml. These contrived specimens were tested with the SARS-CoV-2 NGS Assay and analyzed with the COVID-DX software, which included functionality for mutation calling and lineage calling. The sequencing data was subsampled to 1M and 5M reads (for a total genomic coverage of 5,000x and 25,000x, respectively). The table below summarizes these results:

GENOMIC COVER- AGE	ALPHA REPLI- CATE	800 COPIES/ML		4,000 COPIES/ML		8,000 COPIES/ML		16,000 COPIES/ML		32,000 COPIES/ML		48,000 COPIES/ML	
		# MUTATIONS DETECTED (32 EXPECTED)	PANGOLIN CALL	# MUTATIONS DETECTED (32 EXPECTED)	PANGOLIN CALL	# MUTATIONS DETECTED (32 EXPECTED)	PANGOLIN CALL	# MUTATIONS DETECTED (32 EXPECTED)	PANGOLIN CALL	# MUTATIONS DETECTED (32 EXPECTED)	PANGOLIN CALL	# MUTATIONS DETECTED (32 EXPECTED)	PANGOLIN CALL
5,000x	#1	0	B	19	B.1.1	30	B.1.1.7	32	B.1.1.7	32	B.1.1.7	32	B.1.1.7
	#2	0	B	0	B	0	B	0	B	32	B.1.1.7	12	B.1
25,000x	#1	0	B	31	B.1.1.7	32	B.1.1.7	32	B.1.1.7	32	B.1.1.7	32	B.1.1.7
	#2	0	B	22	B.1.1.7	19	B.1.1	28	B.1.1.7	32	B.1.1.7	32	B.1.1.7

Subsequently, synthetic control RNA material from 4 additional lineages (AY.1, AY.2, B.1.617.2 and P.1; Twist Bioscience) were separately spiked into negative respiratory clinical matrices (NP swabs -clinical samples previously tested negative for SARS-CoV-2) to further estimate an LoD for additional variants based on a limited number of replicates. The variant synthetic controls were spiked into clinical matrices in triplicate at final concentrations of 32,000 copies/mL, 64,000 copies/mL, and 320,000 copies/mL. Subsampling of reads was performed for a total genomic coverage of 300x. The results are shown in the following table:

VARIANT	GISAID ID AND TWIST BIOSCIENCE PRODUCT NUMBER	# CONTROL MUTATIONS EXPECTED	32,000 COPIES/ML		64,000 COPIES/ML		320,000 COPIES/ML	
			AVG # MUTATIONS DETECTED	CORRECT LINEAGE CALL	AVG # MUTATIONS DETECTED	CORRECT LINEAGE CALL	AVG # MUTATIONS DETECTED	CORRECT LINEAGE CALL
AY.1	EPI_ISL_2695467 PN 104538	42	42.3	3/3	42	3/3	41.7	3/3
AY.2	EPI_ISL_2693246 PN 104539	37	35	3/3	35.7	3/3	36.3	3/3
B.1.617.2	EPI_ISL_1544014 PN 104533	37	35.7	3/3	36.7	3/3	37	3/3
P.1	EPI_ISL_792683 PN 104044	31	19.7	2/3	34	3/3	34	3/3

Footnote: Detected mutations were concordant with the synthetic control mutation profile expected. Discordant (extra) mutations detected are considered false.

Based on the results of this study with an expected number of mutations, the average observed error rates across the 3 replicates for each variant were determined as shown in the following table:

AVERAGE OBSERVED # ERRORS/SARS-COV-2 GENOME			
VARIANT	32,000 COPIES/ML	64,000 COPIES/ML	320,000 COPIES/ML
AY.1	4.46E-05	5.58E-05	4.46E-05
AY.2	6.69E-05	8.93E-05	4.46E-05
B.1.617.2	1.12E-04	7.81E-05	6.69E-05
P.1	4.91E-04	3.35E-05	3.35E-05

Due to the prevalence of Omicron, Omicron synthetic control RNA material (B.1.1.529; Twist Bioscience) was also spiked into negative respiratory clinical matrices (NP swabs -clinical samples previously tested negative for SARS-CoV-2). The expected number of mutations within the Omicron synthetic control was 61 mutations as compared to the reference SARS-CoV-2 (NC_045512.2) over the same regions. The Omicron synthetic control was spiked into clinical matrices in duplicate at final concentrations of 133,333 copies/mL and 13,333,333 copies/mL. Subsampling of reads was performed for a total genomic coverage of 300x. For both replicates at each concentration tested, all expected mutations in the synthetic Omicron material were detected. The results are shown in the following table:

OMICRON REPLICATE	133,333 COPIES/ML		13,333,333 COPIES/ML	
	# MUTATIONS DETECTED (61 EXPECTED)	PANGOLIN CALL	# MUTATIONS DETECTED (61 EXPECTED)	PANGOLIN CALL
#1	61	B.1.1.529	61	B.1.1.529
#2	61	B.1.1.529	61	B.1.1.529
#1	61	B.1.1.529	61	B.1.1.529
#2	61	B.1.1.529	61	B.1.1.529

Validation of SARS-CoV-2 PANGO Lineage Determination

SARS-CoV-2 NGS Assay-Specific Sequencing Error Model

A sequencing error model that simulates how sequencing error and ambiguous nucleotides are randomly introduced by the SARS-CoV-2 NGS Assay into a sequenced genome, was estimated based on the sequencing result of 30 clinical samples run at different time periods and in different batches. The error rate for the final error model at each position of the genome was the maximum error rate observed across all 30 clinical samples sequenced, resulting in a population-level error model. At 10X coverage at each genomic position the model estimated 3.73×10^{-1} expected sequencing errors per SARS-CoV-2 genome sequenced and 1.49×10^{-7} expected sequencing errors per SARS-CoV-2 genome sequenced at 300X coverage at each genomic position. At 16X error rate and 30x coverage at each genomic position, the model estimated 2.77 expected sequencing errors per SARS-CoV-2 genome sequenced. Certain regions of the genome had significantly higher error rates. These regions roughly corresponded with regions of known genetic variability in the SARS-CoV-2 genome. The model intentionally treats these as errors despite biological plausibility in order to establish a strict standard..

Simulated Error Study

A simulated error study was conducted to assess the performance of the SARS-CoV-2 NGS Assay to identify samples with PANGO Lineages not tested in analytical studies. 976 high quality SARS-CoV-2 sequences used for training Pangolin were randomly sampled from the set of all training samples and downloaded from GISAID from May 1, 2022, to May 9, 2022, representing 263 unique PANGO lineages and sub-lineages, including 22 unique Omicron sub-lineages and 241 non-Omicron lineages and sub-lineages. The SARS-CoV-2 NGS Assay-specific sequencing error model determined above was used to introduce sequencing errors into these 976 reference sequences to simulate hypothetical sequence output of the assay for these genomes. The error model took two parameters as input: an error amplification rate that controlled how likely errors were relative to the baseline model, and a total genome coverage rate which simulated higher and lower read depths. Each of the 976 sequences were used to produce multiple simulated sequences with 30x, 150x, or 300x coverage and either 1x or 16x the error amplification rate determined in the SARS-CoV-2 NGS Assay-specific sequencing error model. The PANGO lineages of these simulated sequences were then identified with the lineage identification software (Pangolin, using PangoLEARN) used in the SARS-CoV-2 NGS Assay. The lineage identification results of the simulated sequences were subsequently compared to the previously assigned PANGO lineage designations of the reference sequence used to produce the simulated sequences. Importantly, under the worst set of conditions (i.e., 30x total genome coverage + 16x error amplification rate), lineage calling performance was largely unaffected. The summary of these results is shown below.

IN SILICO PERFORMANCE OF SARS-COV-2 NGS ASSAY FOR 976 SEQUENCES SUBMITTED TO GISAID FROM MARCH 2020 TO MARCH 2022

TOTAL GENOME COVERAGE	ERROR AMPLIFICATION RATE	# REFERENCE SEQUENCES TESTED	# FULLY CONCORDANT LINEAGES	% CONCORDANCE 95% CI
30x	1x	976	965	98.9% (98.2%–99.5%)
150x	1x	976	965	98.9% (98.2%–99.5%)
300x	1x	976	965	98.9% (98.2%–99.5%)
30x	16x	976	963	98.7% (97.9%–99.4%)
150x	16x	976	960	98.4% (97.6%–99.2%)
300x	16x	976	960	98.4% (97.6%–99.2%)

Of the 976 genomes, 139 genomes were designated as Omicron sequences. The sub-lineage concordance rates for the simulated Omicron sequences are shown in the following table:

SUB-LINEAGE	TOTAL GENOME COVERAGE	ERROR AMPLIFICATION RATE	# REFERENCE SEQUENCES TESTED	# FULLY CONCORDANT LINEAGES	% CONCORDANCE 95% CI
BA.1	30x	1x	112	108	96.4% (93%-99.9%)
	150x	1x	112	108	96.4% (93%-99.9%)
	300x	1x	112	108	96.4% (93%-99.9%)
	30x	16x	112	108	96.4% (93%-99.9%)
	150x	16x	112	108	96.4% (93%-99.9%)
	300x	16x	112	108	96.4% (93%-99.9%)
BA.1.1	30x	1x	102	98	96.1% (92.3%-99.8%)
	150x	1x	102	98	96.1% (92.3%-99.8%)
	300x	1x	102	98	96.1% (92.3%-99.8%)
	30x	16x	102	98	96.1% (92.3%-99.8%)
	150x	16x	102	98	96.1% (92.3%-99.8%)
	300x	16x	102	98	96.1% (92.3%-99.8%)
BA.2	30x	1x	22	22	100% (95%-100%)
	150x	1x	22	22	100% (95%-100%)
	300x	1x	22	22	100% (95%-100%)
	30x	16x	22	22	100% (95%-100%)
	150x	16x	22	22	100% (95%-100%)
	300x	16x	22	22	100% (95%-100%)
BA.3	30x	1x	5	5	100% (89.9%-100%)
	150x	1x	5	5	100% (89.9%-100%)
	300x	1x	5	5	100% (89.9%-100%)
	30x	16x	5	5	100% (89.9%-100%)
	150x	16x	5	5	100% (89.9%-100%)
	300x	16x	5	5	100% (89.9%-100%)

Controlled Update on SARS-CoV-2 Variant Calling

The identification of emerging SARS-CoV-2 lineages necessitates regular updates to a reference database of lineages. The software will be tested to ensure its continued functionality on database updates. This test will be performed on a number of samples with previously determined lineages spanning a broad base of major lineages. At each database update the set of test samples will be reprocessed to ensure that the lineage designation is the same as previously identified for each sample (with the slight exception of lineage refinements or renaming). This verification test will provide reassurance that there is no regression of the software and the output remains reproducible for a fixed input. As new lineages are identified and processed with the software, examples of these lineages will be added to the test set.

SUPPLEMENTAL INFORMATION

An Illumina platform sample sheet template with appropriate unique barcoding combinations for all 96 reactions found in the SARS-CoV-2 NGS Assay kit can be found in IFU Supplementary Information under the tab labelled as “Template_Sample_Sheet” in the file found at twistbioscience.com/IFU.

Indexing barcode combinations found in the SARS-CoV-2 NGS Assay kit are available in the table below or in excel form in the IFU Supplementary Information under the tab “Barcodes” in the file found at twistbioscience.com/IFU

Twist Universal Adapter System for TruSeq Compatible 10 bp Barcodes, 96 Samples (Plate A):

INDEX	WELL	i7 INDEX REFERENCE FOR SAMPLE SHEET	i5 INDEX REFERENCE FOR SAMPLE SHEET**
1	A01	TATCTTCAGC	CCAATATTCG
2	B01	TGCACGGATA	CGCAGACAAC
3	C01	GGTTGATAGA	TCGGAGCAGA
4	D01	ACTCCTGCCT	GAGTCCGTAG
5	E01	CCGATAGTCG	ATGTTACAGT
6	F01	CAAGATCGAA	TTCGATGGTT
7	G01	AGGCTCCTTC	TATCCGTGCA
8	H01	ATACGGATAG	AAGCGCAGAG
9	A02	AATAGCCTCA	CCGACTTAGT
10	B02	CTGCAATCGG	TTCTGCATCG
11	C02	CCTGAGTTAT	GGAAGTGCCA
12	D02	GACGTCCAGA	AGATTCAACC
13	E02	GAATAATCGG	TTCAGGAGAT
14	F02	CGGAGTGTGT	AAGCGCTCTG
15	G02	TTACCGACCG	ACGCTTGACA
16	H02	AGTGTTCGCC	CATGAAGTGA
17	A03	CTACGTTCTT	TTACGACCTG
18	B03	TGCACACGAA	ATGCAAGCCG
19	C03	CCGATAACTT	CTCCGTATAC
20	D03	TTGGACATCG	GAATCTGGTC
21	E03	AACGTTGAGA	CGGTCGGTAA
22	F03	GGCCAGTGAA	TCTGCTAATG
23	G03	ATGTCTCCGG	CTCTTATTCG
24	H03	GAAGGCGTTC	CACCTCTAGC
25	A04	TGTTCTTAGA	TTACTTACCG
26	B04	CTCTCGAGGT	CTATGCCTTA
27	C04	CTGTACGGTA	GGAAGGTACG
28	D04	CTTATGGCAA	GAGGAGACGT
29	E04	TCCGCATAGC	ACGCAAGGCA
30	F04	GCAAGCACCT	TATCCTGACG
31	G04	GCCTGTCCTA	GAAGACCGCT

CONTINUED TO THE RIGHT

INDEX	WELL	i7 INDEX REFERENCE FOR SAMPLE SHEET	i5 INDEX REFERENCE FOR SAMPLE SHEET**
32	H04	ACTGTCTATC	CAACGTGGAC
33	A05	CGTCCATGTA	TAAGTGCTCG
34	B05	CTAACTGCAA	CACATCGTAG
35	C05	TGCTTGTGGT	ACTACCGAGG
36	D05	TGTAAGCACA	GATGTGTTCT
37	E05	CTCGTTGCGT	AAGTGTGTA
38	F05	GCTAGAGGTG	GGAGAACCAC
39	G05	AAGCGGAGAA	TGTACGAACT
40	H05	AATGACGCTG	GGATGAGTGC
41	A06	TTGGTACGCG	TAGTAGGACA
42	B06	TGAAGGTGAA	ACGCCTCGTT
43	C06	GTAGTGGCTT	CACCGCTGTT
44	D06	CGTAACAGAA	TCTATAGCGG
45	E06	AAGGCCATAA	CCGATGGACA
46	F06	TTCATAGACC	TTCAACATGC
47	G06	CCAACTCCGA	GGAGTAACGC
48	H06	CACGAGTATG	AGCCTTAGCG
49	A07	CCGCTACCAA	TTACCTCAGT
50	B07	CTGAACCTCC	CAGGCATTGT
51	C07	GGCCTTGTTA	GTGTTCCACG
52	D07	TTAACGCAGA	TTGATCCGCC
53	E07	AGGTAGTGCG	GGAGGCTGAT
54	F07	CGTGTAACCT	AACGTGACAA
55	G07	ACTTGTGACG	CACAAGCTCC
56	H07	CCATGCGTTG	CCGTGTTGTC
57	A08	CCTGTAGCG	TTGAGCCAGC
58	B08	ACATACGTGA	GCGTTACAGA
59	C08	CTTGATATCC	TCCAGACATT
60	D08	CAGCCGATGT	TGAACTCTT
61	E08	TCATGCGCTA	ACCTTCTCGG
62	F08	ACTCCGTCCA	AGACGCCAAC

CONTINUED ON THE NEXT PAGE

INDEX	WELL	i7 INDEX REFERENCE FOR SAMPLE SHEET	i5 INDEX REFERENCE FOR SAMPLE SHEET**
63	G08	GACAGCCTTG	CAACCGTAAT
64	H08	CGGTTATCTG	TTATGCGTTG
65	A09	TACTCCACGG	CTATGAGAAC
66	B09	ACTTCCGGCA	AAGTTACACG
67	C09	GTGAAGCTGC	GCAATGTGAG
68	D09	TTGCTCTTCT	CGAAGTCGCA
69	E09	AACGCACGTA	CCTGATTCAA
70	F09	TTACTGCAGG	TAGAACGTGC
71	G09	CCAGTTGAGG	TTCGCAAGGT
72	H09	TGTGCGTTAA	TTAATGCCGA
73	A10	ACTAGTGCTT	AGAACAGAGT
74	B10	CGTGGAACAC	CCATCTGTTC
75	C10	ATGGAAGTGG	TTCGTAGGTG
76	D10	TGAGATCACA	GCACGGTACA
77	E10	GTCCTTGGTG	TGTCAAGAGG
78	F10	GAGCGTGGAA	TCTAAGGTAC
79	G10	CACACGCTGT	GAACGGAGAC
80	H10	TGGTTGTACA	CGCTACCATC
81	A11	ATCACTCACA	TTACGGTAAC
82	B11	CGGAGGTAGA	TTCAGATGGA
83	C11	GAGTTGACAA	TAGCATCTGT
84	D11	GCCGAACTTG	GGACGAGATC
85	E11	AGGCCTCACA	AGGTTCTGTT
86	F11	TCTCTGTTAG	CATACTCGTG
87	G11	TCCGACGATT	CCGGATACCA
88	H11	AGGCTATGTT	ATGTCCACCG
89	A12	CGTTCTCTTG	CACCAAGTGG
90	B12	TTGTCTATGG	TTGAGTACAC
91	C12	GATGGATACA	CGGTTCCGTA
92	D12	CACTTAGGCG	GGAGGTCCTA
93	E12	ACACTGGCTA	CCTGCTTGGA
94	F12	ATCGCCACTG	TTCACGTCAG
95	G12	CTGACGTGAA	AACATAGCCT
96	H12	TCAATCGTCT	TGACATAGTC

**When using Illumina Local Run Manager software for your experimental set up, the i5 barcode sequences in the above table are in the correct orientation. If attempting to use different software, such as bcl2fastq on a Linux server, the above barcode sequences will have to be converted to the reverse complement in order to get appropriate de-multiplexing.

APPENDIX A: NEXTSEQ RUO INSTRUMENT QUALIFICATION

When the SARS-CoV-2 NGS Assay is used in combination with an Illumina NextSeq 500 or NextSeq 550 (Research Use Only (RUO) instruments, as opposed to a NextSeq 550Dx), the platform needs to be tested to assure that the performance of the 500/550 system is qualified within specification of the 550Dx instrument. This should be performed as your first run when using the SARS-CoV-2 NGS Assay on the Illumina NextSeq 500 or NextSeq 550 system.

Three replicates of the following controls should be used to determine if SARS-CoV-2 NGS Assay is being accurately used in combination with the Illumina NextSeq 500 or NextSeq 550:

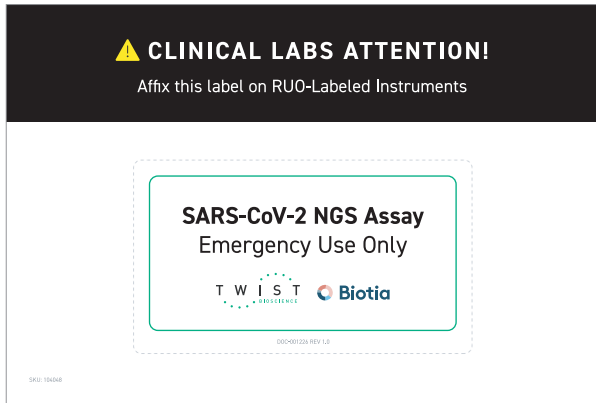
- For the negative extraction control (NEC), aliquot 20 μ l of ATCC VeroE6 cells ($\sim 4.0\text{--}4.5 \times 10^4$ cells total) into 2 ml tubes containing 230 μ l of VTM. Start this process in Module 1 (page 15).
- For the negative template control (NTC), aliquot 250 μ l of VTM into 2.0 ml tubes. Start this process in Module 1 (page 15).
- For the positive control (PC): add 2 μ l of diluted Twist Synthetic SARS-CoV-2 RNA Control 2 (MN908947.3) to 0.5 ml tubes containing 13 μ l of the diluted Agilent Gene Expression Universal Reference RNA (Human). Start this process in Module 2 (page 16).

Once the full assay has been completed, it is necessary to review the data in order to confirm that all replicates meet the following post-sequencing metrics:

CONTROL	EXPECTED RESULT TO PASS
Negative extraction control (NEC)	Valid if 1) SARS-CoV-2 is not detected (output metric < 8.6)
Negative template control (NTC)	Valid if 1) SARS-CoV-2 is not detected (output metric < 8.6)
Positive Twist RNA template control	Valid if 1) SARS-CoV-2 is detected (output metric ≥ 8.6 if < 10,000 bp on SARS-CoV-2 target; output metric ≥ 9.5 if > 10,000 bp on SARS-CoV-2 target) and 2) at least 10,000 reads post minimum read length filtering and at least 10,000 bp on-target bases within GrC38



















If these controls achieve these post-sequencing metrics, then the Illumina NextSeq 500 or NextSeq 550 system is qualified for future runs. After validating the 500/550 system, the “Emergency Use Only” label provided with the SARS-CoV-2 NGS assay should be affixed to the relevant sequencer. If the positive control does not meet these post-sequencing metrics, then the instrument has failed qualification and should not be used for future runs. In addition, any patient specimens evaluated in the first run should be tested with another SARS-CoV-2 assay and results obtained by sequencing should not be reported. Please contact Twist Bioscience technical support at customersupport@twistbioscience.com.

RESEARCH USE ONLY INSTRUMENT LABEL



The Kit includes an Emergency Use Only label. This label should be affixed on the front of any Research Use Only instrument after it has been qualified to use with the SARS-CoV-2 NGS Assay. The qualified instrument should retain this Emergency Use Only label for the duration of the instrument's use with the SARS-CoV-2 NGS Assay under Emergency Use Authorization.

SYMBOLS LEXICON

 European Conformity	 Manufacturer	 Authorized Representative in the European Union	 Lot Number	 Use by	 For In Vitro Diagnostic Use
 Temperature Limit	 Catalog Number	 Consult Instructions for Use	 Number of Tests	 For use with	 Serial Number
 Prescription Use Only	 Unique Device Identification— Device Identifier	 Contains Latex	 Research Use Only	 Single Use Only	 Biohazard

CONTACT INFORMATION, ORDERING, AND PRODUCT SUPPORT



For ordering, product support, or troubleshooting-related questions concerning any molecular biology aspects of this assay, please contact Twist Bioscience at customersupport@twistbioscience.com.



For ordering, product support, or troubleshooting-related questions concerning the analysis pipeline or analytical software, please contact Biotia at customersupport@biotia.io.

REVOKED

LAST REVISED: August 2022

REVISION	DATE	DESCRIPTION
4.0	Aug 2022	<ul style="list-style-type: none"> Updating the IFU to factor in CoV-2 variants.
3.0	Nov 2021	<ul style="list-style-type: none"> Per the request of the FDA on September 23, 2021, labeling updates and performance evaluations related to SARS-CoV-2 viral mutations for authorized tests that are within the scope of Twist's EUA.
2.0	Jun 2021	<ul style="list-style-type: none"> Updated volume values in steps 5.3.8 and 5.3.10 for the addition of sample going on to the sequencing platform. NextSeq Qualification Protocol added as an appendix to assure an RUO sequencing platform runs as a Dx platform would.



STOP. YOU MUST READ THIS BEFORE PROCEEDING.

Thank you for purchasing the SARS-CoV-2 NGS Assay.

BOX 1 OF 3 CONTAINS THE FOLLOWING:

1x Twist Library Preparation EF Kit 1, 96 Samples
Manual Kit EUA SKU: 102873

**1x Twist Universal Adapter System:
TruSeq Compatible, 96 Samples Plate A**
Manual Kit EUA SKU: 102876

1x Twist Universal Blockers, 12 Reactions
Manual Kit EUA SKU: 102881

1x Twist Fast Hybridization Reagents, 12 Reactions
Manual Kit EUA SKU: 102883

1x Twist SARS-CoV-2 Research Panel, 12 Reactions, Kit
Manual Kit EUA SKU: 102880

SHIPPED ON DRY ICE, STORE AT -25°C TO -15°C

PRODUCT INFORMATION

- IVD, Rx-only, For use under EUA only
- The full instructions are not included here
- Please contact 800-719-0671 if you require a printed copy of the Instructions for Use free of charge
- Instructions/webpage links to the full labeling documents can be found here twistbioscience.com/IFU
- This product has not been FDA cleared or approved, but has been authorized for emergency use by FDA under an EUA for use by authorized laboratories
- This product has been authorized only for the detection of nucleic acid from SARS-CoV-2, along with the identification and differentiation of SARS-CoV-2 PANGO lineages and the identification of specific SARS-CoV-2 genomic mutations, not for any other viruses or pathogens
- The emergency use of this product is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of in vitro diagnostics for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Federal Food, Drug and Cosmetic Act, 21 U.S.C. § 360bbb-3 (b)(1), unless the declaration is terminated or authorization is revoked sooner



To learn more:

- Instructions for Use (IFU): twistbioscience.com/IFU
- Product Specification Sheet: twistbioscience.com/NGSassay
- Patient Fact Sheet: twistbioscience.com/patientfactsheet
- Healthcare Provider Fact Sheet: twistbioscience.com/HCPfactsheet



Contact customersupport@biotia.io for software specific support

Biotia COVID-DX (v1) software has been built to reliably analyze the SARS-CoV-2 NGS Assay built by Twist Bioscience. Each Twist kit includes access to bioinformatic analysis for 101 samples (for a 96-sample kit). Register through the Biotia user portal biotia.io and enter your unique order number, which will be emailed to you, to receive your Biotia analysis credits. Credits for Biotia COVID-DX (v1) expire one year after Twist kit purchase. Please visit twistbioscience.com/IFU to access Instructions for Use (IFU).



STOP. YOU MUST READ THIS BEFORE PROCEEDING.

Thank you for purchasing the SARS-CoV-2 NGS Assay.

BOX 2 OF 3 CONTAINS THE FOLLOWING:

2x Twist Library Preparation Kit 2, 96 Samples
Manual Kit EUA SKU: 102874

1x Twist Binding and Purification Beads, 12 Reactions
Manual Kit EUA SKU: 102882

1x Twist Fast Wash Buffers, 12 Reactions
Manual Kit EUA SKU: 102884

103031 Biotia COVID-DX (v1.0)
SKU#: 104270

SHIPPED AMBIENT, STORE AT 2°C TO 8°C

PRODUCT INFORMATION

- IVD, Rx-only, For use under EUA only
- The full instructions are not included here
- Please contact 800-719-0671 if you require a printed copy of the Instructions for Use free of charge
- Instructions/webpage links to the full labeling documents can be found here twistbioscience.com/IFU
- This product has not been FDA cleared or approved, but has been authorized for emergency use by FDA under an EUA for use by authorized laboratories
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- The emergency use of this product is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of in vitro diagnostics for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Federal Food, Drug and Cosmetic Act, 21 U.S.C. § 360bbb-3 (b)(1), unless the declaration is terminated or authorization is revoked sooner



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STOP. YOU MUST READ THIS BEFORE PROCEEDING.

Thank you for purchasing the SARS-CoV-2 NGS Assay.

BOX 3 OF 3 CONTAINS THE FOLLOWING:

1x Twist Synthetic SARS-CoV-2 RNA Control 2 (MN908947.3)
Manual Kit EUA SKU: 102875

SHIPPED ON DRY ICE, STORE AT -90°C TO -70°C

PRODUCT INFORMATION

- IVD, Rx-only, For use under EUA only
- The full instructions are not included here
- Please contact 800-719-0671 if you require a printed copy of the Instructions for Use free of charge
- Instructions/webpage links to the full labeling documents can be found here twistbioscience.com/IFU
- This product has not been FDA cleared or approved, but has been authorized for emergency use by FDA under an EUA for use by authorized laboratories
- This product has been authorized only for the detection of nucleic acid from SARS-CoV-2, along with the identification and differentiation of SARS-CoV-2 PANGO lineages and the identification of specific SARS-CoV-2 genomic mutations, not for any other viruses or pathogens
- The emergency use of this product is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of in vitro diagnostics for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Federal Food, Drug and Cosmetic Act, 21 U.S.C. § 360bbb-3 (b)(1), unless the declaration is terminated or authorization is revoked sooner



To learn more:

- Instructions for Use (IFU): twistbioscience.com/IFU
- Product Specification Sheet: twistbioscience.com/NGSassay
- Patient Fact Sheet: twistbioscience.com/patientfactsheet
- Healthcare Provider Fact Sheet: twistbioscience.com/HCPfactsheet



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