SARS-CoV-2 NGS Assay—RUO

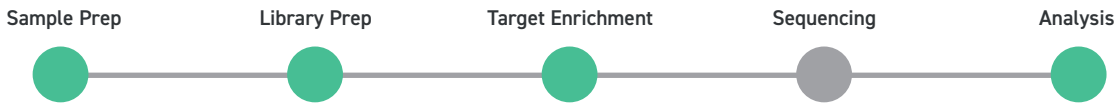
Using the Twist Target Enrichment Workflow and the Biotia COVID-DX (v1.0) Software

FOR RESEARCH-USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

The SARS-CoV-2 NGS Assay is a highly sensitive nucleic acid hybridization capture-based research-use only assay used for the detection, characterization, and environmental monitoring 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 (v1.0)).

This assay is intended for the qualitative detection of the SARS-CoV-2 virus from nasopharyngeal (NP) swabs, oropharyngeal (OP) swabs, anterior nasal swabs, mid-turbinate nasal swabs, nasopharyngeal wash/aspirates, nasal aspirates, and bronchoalveolar lavage (BAL) specimens for research purposes only. The SARS-CoV-2 NGS Assay enriches for and detects all nucleic acid sequences by hybrid capture target enrichment from the SARS-CoV-2 virus, as opposed to only identifying certain regions. This technology requires a hybridization time of 2 hours, estimating a sensitivity level as few as 800 viral copies per ml in each specimen.

The Biotia COVID-DX (v1.0) software provides a research-oriented report that includes the full sequence of the SARS-CoV-2 virus, enabling improved understanding of mutations, genetic variations, and the evolution of the virus as it is transmitted. FASTQ files (sequencing output) can be generated in laboratories and submitted to Biotia COVID-DX (v1.0), a cloud-based software, to generate research-use only reports. The purchase of each SARS-CoV-2 NGS Assay kit includes credits for COVID-DX (v1.0) bioinformatic analysis of each sample. To receive Biotia analysis credits, simply register through the Biotia User Portal via www.biotia.io and enter the unique order number emailed to you. These credits provide access to run samples through the report generation component of the software.



Twist NGS workflow. The complete NGS workflow takes you from sample preparation to NGS sequencing and data analysis.

PUBLISHED: December 2020

PROTOCOL COMPONENTS

This protocol provides recommendations for running the SARS-CoV-2 research use only assay to detect, characterize, and monitor the SARS-CoV-2 virus in samples collected from research subjects. It is not meant to provide instructions for clinical diagnosis.

Read the product packaging and storage recommendations carefully for each component, and store components as recommended below immediately upon arrival.

PART NUMBER	NAME	DESCRIPTION	STORAGE
TWIST PROBE PANELS AND BIOTIA ANALYSIS SOFTWARE			
103564: 2 rxn 103566: 12 rxn 103567: 96 rxn	Twist SARS-CoV-2 Research Panel + Biotia COVID-DX (v1.0)	SARS-CoV-2 detection panel and analysis software	–25 to –15°C
TWIST VIRAL CONTROLS (ORDERED SEPARATELY)			
See Appendix B	Twist Synthetic SARS-CoV-2 RNA Controls	Synthetic SARS-CoV-2 RNA control	–90 to –70°C
101059: 16 rxn 101058: 96 rxn	Twist Library Preparation EF Kit	Reagents for library construction	
	Twist Library Preparation EF Kit 1	5x Fragmentation Enzyme 10x Fragmentation Buffer DNA Ligation Mix DNA Ligation Buffer Amplification Primers, ILMN (Tubes 100220, 100583 are not required when used with universal adapters)	–25 to –15°C
	Twist Library Preparation Kit 2	DNA Purification Beads	2–8°C
100401: 16 rxn 100573: 96 rxn	Twist Library Preparation Kit 2	DNA Purification Beads (as a Standalone Product, Bead Purification is also needed during cDNA Synthesis)	2–8°C
TWIST FAST HYBRIDIZATION REAGENTS (ORDERED SEPARATELY)			
101307: 16 rxn 101308, 101309, 101310, 101311: 96 rxn	Twist Universal Adapter System - TruSeq Compatible	Twist Universal Adapters and Twist UDI Primers, provides unique dual-indexed combinations with 1 reaction per index pair.	–25 to –15°C
100856: 2 rxn 100578:12 rxn 100767: 96 rxn	Twist Universal Blockers	For the prevention of nonspecific capture: Universal Blockers Blocking Solution	–25 to –15°C
101262: 2 rxn 100983: 12 rxn 100984: 96 rxn	Twist Binding and Purification Beads	For target enrichment and purification: Streptavidin Binding Beads DNA Purification Beads	2–8°C
101278: 2 rxn 101174: 12 rxn 101175: 96 rxn	Twist Fast Hybridization and Wash Kit (2 Boxes)	For target enrichment with Twist Fast hybridization:	
	Twist Fast Hybridization Reagents (Box 1 of 2)	Fast Hybridization Mix Hybridization Enhancer Amplification Primers	–25 to –15°C
	Twist Fast Wash Buffers (Box 2 of 2)	Fast Binding Buffer Fast Wash Buffer 1 Wash Buffer 2	2–8°C

*This product is for **research-use only**.*

LEGAL

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Twist Bioscience received ISO 13485:2016 certification for our quality management system governing the design and manufacture of our NGS TE Panels.

INTENDED USE

The SARS-CoV-2 NGS Assay is a highly sensitive nucleic acid hybridization capture-based research-use only assay used for the detection, characterization, and environmental monitoring of the SARS-CoV-2 virus. This product is for research use only. It is not intended for the diagnosis, prevention, or treatment of a disease or condition. Twist Bioscience assumes no liability regarding use of the product for applications in which it is not intended.

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TABLE OF CONTENTS

MATERIALS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

The following materials are required (but not included) to perform the SARS-CoV-2 NGS Assay:

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
Direct-zol DNA/RNA Miniprep 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 (S1230S)
Gene Expression Universal Reference RNA (Human)	Agilent Technologies (740000-41)
VERO C1008 [Vero 76, clone E6, Vero E6]	ATCC (optional)
Viral Transport Media (VTM)	— (optional)
KAPA HiFi HotStart ReadyMix	Kapa Biosystems
Ethanol (200 proof)	—
Nuclease-free water	—
Qubit RNA High Sensitivity Quantification Assay or equivalent	Thermo Fisher Scientific
Qubit dsDNA Broad Range Quantitation Assay or equivalent	Thermo Fisher Scientific
Qubit dsDNA High Sensitivity Quantitation Assay or equivalent	Thermo Fisher Scientific
Agilent High Sensitivity DNA Kit	Agilent Technologies
Agilent DNA 7500 Kit	Agilent Technologies
2.0-ml microcentrifuge tubes	VWR
1.5-ml microcentrifuge tubes	VWR
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well thermal cycling plates	VWR
Adhesive PCR plate foils	—
PCR cryogenic rack	—
1.5-ml microcentrifuge tube compatible magnetic stand	Beckman Coulter
96-well thermal cycling plate compatible magnetic plate	Alpaqua, Permagen Labware
EQUIPMENT	
Pipette and tips	—
Vortex mixer	—
Benchtop mini centrifuge for 1.5-ml microcentrifuge tube	—
Vacuum concentrator with plate rotor	—
Thermal cycler (96-well) with heated lid	—
Fluorometer (Qubit 3.0) or equivalent	Thermo Fisher Scientific
2100 Bioanalyzer Agilent Technologies	Agilent Technologies
Thermomixer for 1.5-ml microcentrifuge tube (2x)	Eppendorf
Centrifuge with 96-well plate rotor	—
Centrifuge for 1.5-ml microcentrifuge tube	—

GENERAL NOTES AND PRECAUTIONS

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.

For best results, read this document before performing the protocol and follow the instructions provided. Twist cannot guarantee performance if modifications are made to the protocol.

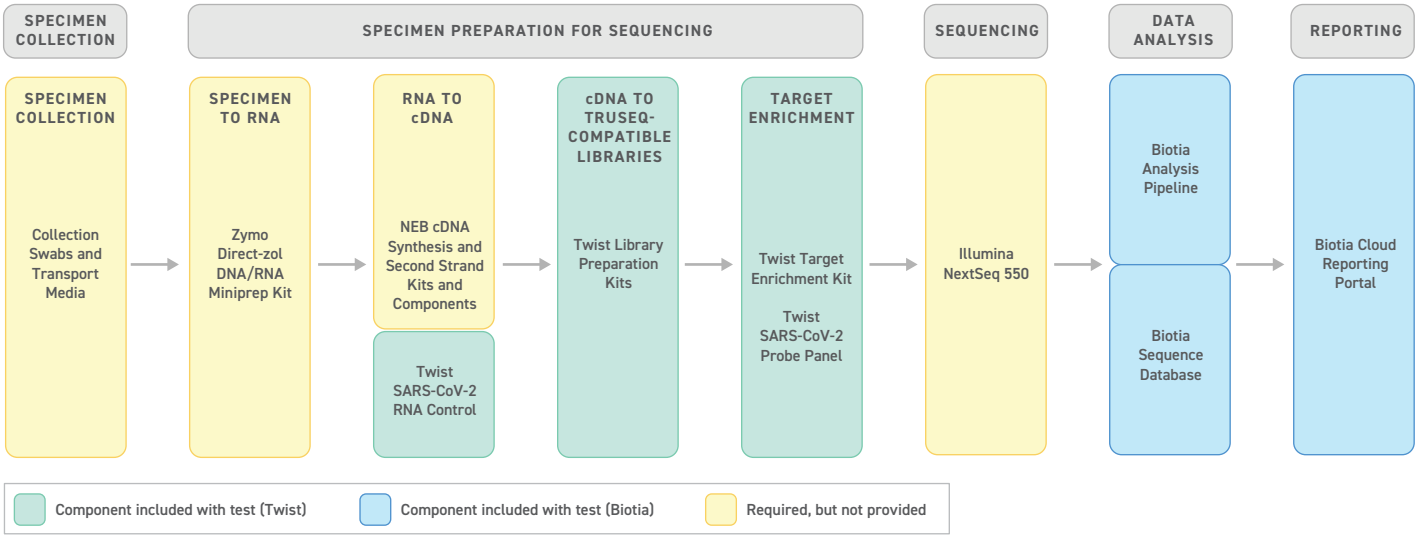

Figure 1: Workflow Overview Diagram. Components for the SARS-CoV-2 NGS Assay are specified as included by Twist (blue) or Biotia (green), and required but not provided (yellow). General sample and reagent processing steps are listed above (grey).

- Optimal performance of this research use only assay requires appropriate specimen collection, storage, and transport. Detection of the SARS-CoV-2 virus may be affected by sample collection methods, individual factors (e.g., presence of symptoms), and/or stage of infection.
- This library preparation method may yield more material than needed for target enrichment. Excess product can be stored at –20°C for later use.
- Test the compatibility of your thermal cycler and PCR tubes by incubating at 95°C for up to 5 minutes to ensure the PCR tubes do not crack under heat and pressure. Adjust the tightness of the thermal cycler lid and/or use a spacer specific to the thermal cycler model.
- This protocol details different methods for mixing reagents (gentle pipetting, flicking or tapping, vortexing), depending on the volume, vessel, and reagents involved.
- The Fast Hybridization Mix is a viscous reagent. Pipette slowly to ensure accuracy. 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.
- For technical support, contact NGSsupport@twistbioscience.com

IMPORTANT NOTES

- Store all RNA samples at –80°C when not in use. Keep on ice at all times when in use.
- Input RNA or purification resuspensions should be suspended in nuclease-free water.
- 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.
- 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.
- Prior to library preparation, it is important to remove all cations and chelators as these may affect the initial fragmentation reaction.
- Using higher cDNA input will increase library diversity and reduce duplication rate, but optimization of the following steps in library preparation may still be required to achieve optimal performance.
  1. Incubation time for fragmentation at 32°C (Step 3.1.2, page 14)
  2. Amount of Twist Universal Adapter (Step 3.2.4, page 15)
  3. Incubation time for ligation reaction (Step 3.2.8, page 15)
- Measuring DNA concentration by absorbance at 260 nm is not recommended. Use the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay to accurately quantify input purified cDNA.
- 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.
- 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 government safety standards.
- For technical support, contact customersupport@twistbioscience.com

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 an RNase/DNase-free environment. Thoroughly decontaminate work areas with an 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.
- 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.**

PROTOCOL OVERVIEW

This protocol begins with nucleic acid extraction and generates cDNA from RNA. The dsDNA biotin-bound hybrid capture research panel used in the assay is designed to enrich for all evolved virus sequences in upper respiratory specimens (nasopharyngeal swabs) from individuals with symptoms indicative of COVID-19. This capture-based research assay utilizes cDNA synthesis from template viral and human RNA sequence in an individual's specimen, producing amplified libraries from the cDNA using a PCR thermal cycler. The SARS-CoV-2 virus sequence, regardless of strain, 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 thermal cycler and sequenced by an Illumina-based NGS platform (NextSeq 550 system). The Biotia COVID-DX (v1.0) software analyzes sequencing results to provide a research-oriented report that includes the presence of the SARS-CoV-2 virus in each sample. Access to the Biotia COVID-DX (v1.0) software, data analysis, and report generation is included with the purchase of each SARS-CoV-2 NGS Assay kit. Customers receive Biotia analysis credits via the unique request number emailed to them upon purchase. This protocol allows for end-to-end workflow (Modules 1 to 5 and Pipeline Analysis) in up to 50 hours total. Sequencing time can take up to 18 hours, while the bioinformatics pipeline analysis takes approximately 2 hours.

MODULE	STAGE	TIME
VIRAL SPECIMENS AND CONTROLS		
MODULE 1: Nucleic Acid Extraction	**Stage 1: Nucleic Acid Extraction** *Purified RNA Samples*	7 hours
15 µl OF PURIFIED RNA SAMPLES		
MODULE 2: cDNA Synthesis	**Stage 1: First-Strand Synthesis** *Single-Stranded cDNA Material*	1.5 hours
	Stage 2: Second-Strand Synthesis *Double-Stranded cDNA Material*	1 hour
	Stage 3: cDNA Clean-Up *cDNA Material for Library Preparation*	2 hours
50 NG OF PURIFIED cDNA MATERIAL		
MODULE 3: DNA LIbrary Preparation	**Stage 1: Enzymatic Fragmentation, End Repair, and dA-tailing** *dA-Tailing DNA Fragments*	1.5 hours
	Stage 2: Twist Universal Adapter Ligation *cDNA Libraries with Universal Adapters*	1.5 hours
	Stage 3: Ligation Clean-Up *Purified cDNA Libraries*	1 hour
	Stage 4: PCR Amplification *Amplified Indexed Libraries*	1 hour
	Stage 5: PCR Product Clean-Up *Purified Indexes Libraries*	2 hours
187.5 NG OF AMPLIFIED AND PURIFIED INDEXES LIBRARIES		
MODULE 4: SARS-CoV-2 Capture	**Stage 1: Pooling Strategy** *Indexed Library Pool*	3 hours
	Stage 2: Target Enrichment (Hybridization) *Hybridized Targets in Solution*	2 hours
	Stage 3: Streptavidin Binding of Targets and Hybridization Washes *Captured Targets on Beads*	2 hours
	Stage 4: Post Capture PCR Amplification *Enriched Libraries*	0.5 hours
	Stage 5: Post Capture Library Clean-Up *Purified Enriched Libraries*	1 hour
ILLUMINA NGS PLATFORM, SINGLE-END READ RUN		

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 (~4.0–4.5 x 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 microcentrifuge 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 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 x 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 x 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 x g to ensure complete removal of the wash buffer. Carefully transfer the column into a 1.5-ml nuclease-free microcentrifuge tube.

1.1.8 To elute RNA, add 50 µl of nuclease-free water directly to the column matrix and centrifuge at 16,000 x 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.

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:

Recommended Controls:

- Dilute the Agilent Gene Expression Universal Reference RNA (Human) to 3.85 ng/µl.
- 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 to 50 copies/µl. For Twist Synthetic SARS-CoV-2 RNA Control strains, refer to Appendix B.
- For the positive control (PC), add 2 µl of diluted Twist Synthetic SARS-CoV-2 RNA Control 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.

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 50 µl of the diluted NEB Random Primers into clean microcentrifuge tubes for future use. 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 clean 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 µl
NEBNext Second Strand Synthesis Reaction Buffer	8 µl
NEBNext Second Strand Synthesis Enzyme Mix	4 µl

2.2.2 Add 30 µl 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 µl 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 µl (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 µl 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 µl 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 overdry the bead pellet.

2.3.12 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.

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 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, without disturbing the bead pellet.

2.3.16 Place samples on ice and proceed to QC the cDNA by quantifying with Qubit DNA High Sensitivity Quantification Assay.

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 a 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 clean 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 pipet, 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 overdry 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. For index selection and multiplexing refer to the pooling guidelines in the Appendix A.

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 pipet, 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 overdry 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. Quantify and validate the size range of each library using the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay and Agilent DNA 7500 Assay. Average fragment length should be 250–450 bp using a range setting of 150–1,000 bp.

THIS IS A SAFE STOPPING POINT.
STORE SAMPLES AT –20°C IF NOT IMMEDIATELY PROCEEDING TO MODULE 4.

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 clean thin-walled PCR 0.2 ml 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 a 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 µl 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 µl 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 µl of Twist Fast Wash Buffer 1 and preheat to 68°C. For each hybridization reaction, aliquot 700 µl 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 µl Twist Streptavidin Binding Beads to a 1.5-ml microcentrifuge tube. Prepare one tube for each hybridization reaction.

4.3.6 Add 200 µl 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 not to 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 µl 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 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 µl 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 not to disturb the bead pellet.

4.3.19 Remove the tube(s) from the magnetic stand and add an additional 200 µl 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 µl) 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 not to disturb the bead pellet.

4.3.24 Remove the tube(s) from the magnetic stand and add 200 µl 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 and discard the clear supernatant. Make sure not to 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 pipet. 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 a thermal cycler with the following conditions. Set the heated lid to 105°C.

	STEP	TEMPERATURE	TIME	NUMBER CYCLES
1	Initialization	98°C	45 seconds	1
2	Denaturation	98°C	15 seconds	16
	Annealing	60°C	30 seconds	
	Extension	72°C	30 seconds	
3	Final Extension	72°C	1 minute	1
4	Final Hold	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 clean 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 µl 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 µl (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 µl 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 µl pipet. 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 overdry.

4.5.11 Remove the plate or tubes from the magnetic plate and add 32 µl 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 µl 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. Validate and quantify each enriched library using an Agilent Bioanalyzer High Sensitivity DNA Kit and a Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay.

Proceed with the Illumina sequencing platform of your choice using a single-end read run. Biotia COVID-DX software cannot be used with a paired-end read run.

STORE SAMPLES AT –20°C IF NOT IMMEDIATELY SEQUENCED.

DESCRIPTION OF BIOTIA USER PORTAL: DATA TRANSFER AND REPORTING

Biotia COVID-DX (v1.0) software has been built to analyze the SARS-CoV-2 NGS-Assay, generating a research-use only 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 (v1.0) Software

After sequencing, users can upload their FASTQ files through our simple user interface (Biotia Portal lined through www.biotia.io) and receive a research-oriented report to determine presence/absence of the SARS-CoV-2 virus with genetic variants mapped across the viral genome and phylogenetic analysis.

The research-use only SARS-CoV-2 NGS Assay includes access to bioinformatic analysis for each of your samples. To receive your Biotia analysis credits, register on the Biotia Portal by going to www.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 (v1.0) expire one (1) year after purchasing the SARS-CoV-2 NGS Assay.

Biotia Portal Registration

- Visit www.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'.
- Based on the provided data, while we review, the system will automatically send you a link via email.
- 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. Here, 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 (e.g., 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

- Researchers and reference laboratory personnel can register a new order by selecting 'COVID-DX' in the Menu Bar.
- Choose the Researcher/Lab Personnel email 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 order number, which is emailed to you with your purchase, to access your credits for the Biotia COVID-DX (v1.0) 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. Note: you may have more than one FASTQ file per sample depending on your sequencing lane design. Upload 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 'Save' then 'Submit'. 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 Researchers/Lab Personnel on the Biotia Portal.
- Users will receive an email notifying them that their reports are ready for review and download.

DESCRIPTION OF COVID-DX (V1.0) SOFTWARE AND RESEARCH-USE ONLY REPORT

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

Pre-processing and Quality Control

- The program cutadapt is implemented first to trim adapter sequences, and remove low-quality bases (<Q30) from input FASTQ files, using the standard Illumina adapter sequence "AGATCGGAAGAGC" and a minimum read length of 20 bp (after low-quality bases have been trimmed). Subsequently, trimmed FASTQs are aligned against the genomes of 26 other viral and bacterial respiratory pathogens, as well as the human genome, to eliminate the potential for off-target reads that spuriously align to our reference SARS-CoV-2 virus (NC_045512.2).
- The 26 genomes are combined into one large FASTA file that is indexed with the Bowtie2-build. FASTQs are split for parallelization and aligned using Bowtie2 run in local mode to perform alignments of the smaller FASTQs against the combined FASTA.
- The research use only 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 inaccurate detection due to cross-hybridization of the probes 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 https://github.com/broadinstitute/picard/releases/tag/2.23.0.
- The depth of reads at each site across the SARS-CoV-2 genome is finally calculated using samtools-depth and is then passed on for further analysis.
- A cut-off limit was set and samples with lower than 10,000 total reads are assigned as negative. Samples with higher than 50,000,000 reads are subsampled to 50,000,000 reads before further analysis.

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.
- We devised a metric to compute 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 9.6 if samples had more than 10,000 bases on target. Metrics were calculated using the R statistical software package (v4.0.1).

Genetic Variants

- The BAM file is subsampled to a maximum of 500x coverage at a given site within the SARS-CoV-2 genome because coverage can exceed 8,000x, which is computationally prohibitive for variant calling tools. Variants are called in the subsampled BAM file using GATK GenotypeVCF and HaplotypeCaller.
- If a sample is identified as negative, variants are not reported.

Coverage Plot

- To visualize the depth of reads that mapped across the SARS-CoV-2 genome, we generated a coverage plot using the R software and the data generated by samtools-depth.
- The diagram also demonstrates the positions of the genes within the SARS-CoV-2 genome so that users can see which regions of the genome, if any, have low or missing coverage.

Phylogenetic Analysis and Geography

- The test sample HaplotypeCaller VCF file is merged with a NextStrain VCF file with 3,365 samples using BCFtools. A phylogenetic tree is created using R-packages SNPRelate and gdsfmt. The tree was built using hierarchical cluster analysis on Identify by State (IBS) genetic pairwise distances matrix built on GISAID variants reported (version 23-April-2020).
- Clade geographic data were calculated using GISAID (version 24-June-2020) data to provide the percentage of samples sequenced in each country that are categorized into each clade among all samples of each clade.

HS Metrics Reporting

- Picard CollectHSMetrics, GATK CallableLoci, and FASTQC are also used to summarize capture performance.

Report Generation

- Outputs are consolidated in a combination of PDF and XML formats, which are processed by an Azure Function to generate a final research report for distribution to the requester. The report states if SARS-CoV-2 and variants were detected. Furthermore, it provides information on coverage plot, phylogenetic analysis, and HS metrics. If a sample is identified as negative, variants are not reported. If a sample has a viral level that is prohibitive for classification due to depth of coverage, then coverage plots, and phylogenetic trees are not reported. If a sample has a viral level that has extreme depth that falls above our model range, “Very high viral level” will be reported.
- Outputs will include FASTA files to support further research.

CONTROLS

The following controls are used during the protocol:

DESCRIPTION OF CONTROL	TYPE OF CONTROL	CONTROL TO SAMPLE RATIO	AMOUNT USED	USED TO MONITOR	PERFORMANCE DESCRIPTION
Viral transport media (VTM)	Negative/ no template control (NTC)	One control for 96 test samples	250 µl	NTC is used to eliminate the possibility of sample contamination on the assay run and is used with each extraction batch through sequencing.	Negative for all targets detected
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.	Negative for all targets detected
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	50 copies/µl, 100 copies total	PC is used 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 in vitro transcription using the DNA template as reference.	Positive for the targets detected
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.	Negative for SARS-CoV-2 targets

If any control does not perform as described above, the run is considered invalid. These are the recommended controls, however other controls can be used during the process. **Please keep in mind you need to include NTC, NEC, PC, and IC to be able to generate research data using the COVID-DX software.**

The Biotia COVID-DX (v1.0) software will provide a research-oriented report including the detection of the SARS-CoV-2 virus and genetic variants, coverage plot, phylogenetic analysis, and HS metrics.

APPENDIX A: ADAPTER SEQUENCES AND POOLING GUIDELINES

UDI SEQUENCES

For a complete guide of the Twist UDI sequences please refer to PDF document DOC-001129 or Excel file DOC-001130. Both files are available for download at twistbioscience.com/resources.

POOLING GUIDELINES

Twist UDI primers are base balanced by column for next generation sequencing. When pooling unique dual-indexed libraries for 8-plex hybridization, it is recommended that libraries be selected from a single column. Multiple columns may be selected in any desired combination across a single plate or multiple plates for sequencing.

Table 1. Twist UDI primer plate layouts and pooling guidelines.
Twist Universal Adapter System: TruSeq Compatible, 16 Samples (101307)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9										
B	2	10										
C	3	11										
D	4	12										
E	5	13										
F	6	14										
G	7	15										
H	8	16										

*PLEASE NOTE: The indexes in the 16-sample plate are not the same for 96 samples (Plate A).

APPENDIX A: ADAPTER SEQUENCES AND POOLING GUIDELINES

Twist Universal Adapter System: TruSeq Compatible, 96 Samples, Plates A to D (101308, 101309, 101310, 101311)

Plate A.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Plate B.

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	105	113	121	129	137	145	153	161	169	177	185
B	98	106	114	122	130	138	146	154	162	170	178	186
C	99	107	115	123	131	139	147	155	163	171	179	187
D	100	108	116	124	132	140	148	156	164	172	180	188
E	101	109	117	125	133	141	149	157	165	173	181	189
F	102	110	118	126	134	142	150	158	166	174	182	190
G	103	111	119	127	135	143	151	159	167	175	183	191
H	104	112	120	128	136	144	152	160	168	176	184	192

Plate C.

	1	2	3	4	5	6	7	8	9	10	11	12
A	193	201	209	217	225	233	241	249	257	265	273	281
B	194	202	210	218	226	234	242	250	258	266	274	282
C	195	203	211	219	227	235	243	251	259	267	275	283
D	196	204	212	220	228	236	244	252	260	268	276	284
E	197	205	213	221	229	237	245	253	261	269	277	285
F	198	206	214	222	230	238	246	254	262	270	278	286
G	199	207	215	223	231	239	247	255	263	271	279	287
H	200	208	216	224	232	240	248	256	264	272	280	288

Plate D.

	1	2	3	4	5	6	7	8	9	10	11	12
A	289	297	305	313	321	329	337	345	353	361	369	377
B	290	298	306	314	322	330	338	346	354	362	370	378
C	291	299	307	315	323	331	339	347	355	363	371	379
D	292	300	308	316	324	332	340	348	356	364	372	380
E	293	301	309	317	325	333	341	349	357	365	373	381
F	294	302	310	318	326	334	342	350	358	366	374	382
G	295	303	311	319	327	335	343	351	359	367	375	383
H	296	304	312	320	328	336	344	352	360	368	376	384

END OF APPENDIX A

APPENDIX B: TWIST SYNTHETIC SARS-COV-2 CONTROLS

Each viral control is made synthetically with Twist's silicon platform and in vitro transcription. Each control is broken up between six unique 5 kb fragments, spanning the viral genome.

CATALOG #	NAME	DESCRIPTION	NUCLEIC ACID SPECIES	STORAGE
102019	Twist Synthetic SARS-CoV-2 RNA Control 1 (MT007544.1)	Synthetic SARS-CoV-2 RNA controls	ssRNA	–90 to –70°C
102024	Twist Synthetic SARS-CoV-2 RNA Control 2 (MN908947.3)	Synthetic SARS-CoV-2 RNA controls	ssRNA	–90 to –70°C
102860	Twist Synthetic SARS-CoV-2 RNA Control 3 (LC528232.1)	Synthetic SARS-CoV-2 RNA controls	ssRNA	–90 to –70°C
102862	Twist Synthetic SARS-CoV-2 RNA Control 4 (MT106054.1)	Synthetic SARS-CoV-2 RNA controls	ssRNA	–90 to –70°C
102917	Twist Synthetic SARS-CoV-2 RNA Control 5 (MT188340)	Synthetic SARS-CoV-2 RNA controls	ssRNA	–90 to –70°C
102918	Twist Synthetic SARS-CoV-2 RNA Control 6 (MT118835)	Synthetic SARS-CoV-2 RNA controls	ssRNA	–90 to –70°C
103087	Twist Synthetic SARS-CoV-2 RNA Control 7 (EPI_ISL_418227)	Synthetic SARS-CoV-2 RNA controls	ssRNA	–90 to –70°C
103511	Twist Synthetic SARS-CoV-2 RNA Control 8 (MT066176)	Synthetic SARS-CoV-2 RNA controls	ssRNA	–90 to –70°C
103512	Twist Synthetic SARS-CoV-2 RNA Control 9 (MT152824)	Synthetic SARS-CoV-2 RNA controls	ssRNA	–90 to –70°C
103513	Twist Synthetic SARS-CoV-2 RNA Control 10 (EPI_ISL_414648)	Synthetic SARS-CoV-2 RNA controls	ssRNA	–90 to –70°C
1035114	Twist Synthetic SARS-CoV-2 RNA Control 11 (EPI_ISL_417739)	Synthetic SARS-CoV-2 RNA controls	ssRNA	–90 to –70°C
1035115	Twist Synthetic SARS-CoV-2 RNA Control 12 (EPI_ISL_420244)	Synthetic SARS-CoV-2 RNA controls	ssRNA	–90 to –70°C
1035133	Twist Synthetic SARS-CoV-2 RNA Control 13 (EPI_ISL_421184)	Synthetic SARS-CoV-2 RNA controls	ssRNA	–90 to –70°C

END OF APPENDIX B

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 software, please contact Biotia at customersupport@biotia.io.

LAST REVISED: December 03, 2021

REVISION	DATE	DESCRIPTION
5.0	Dec 03, 2021	Removal of ISO 9001:2015
4.0	Mar 04, 2021	Pg. 11, Reagents: Aliquot volume updated from 500 µl to 50 µl
3.0	Feb 24, 2021	Minor grammatical edit
2.0	Jan 21, 2021	Removed sentence “full list of 26 genomes will be provided”