

Illumina COVIDSeq Test Instructions for Use

FOR IN VITRO DIAGNOSTIC USE

FOR USE UNDER AN EMERGENCY USE AUTHORIZATION (EUA) ONLY

FOR PRESCRIPTION USE ONLY

Intended Use

The Illumina® COVIDSeq™ Test is a Next-Generation Sequencing (NGS) *in vitro* diagnostic test on the Illumina NovaSeq 6000 Sequencing System, NextSeq 500 Sequencing System, NextSeq 550 Sequencing System, or NextSeq 550Dx Instrument intended for the qualitative detection of SARS-CoV-2 RNA from nasopharyngeal (NP) swabs, oropharyngeal (OP) swabs, anterior nasal swabs, mid-turbinate nasal swabs, nasopharyngeal wash/aspirates, nasal aspirates, and bronchoalveolar lavage (BAL) specimens from individuals suspected of COVID-19 by their healthcare provider.

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.

Results are for the identification of SARS-CoV-2 RNA. The SARS-CoV-2 RNA is generally detectable in respiratory specimens during the acute phase of infection. Positive results are indicative of the presence of 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. Laboratories within the United States and its territories are required to report all positive 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 Illumina® COVIDSeq™ Test is intended for use by qualified and trained clinical laboratory personnel specifically trained in the use of the NovaSeq 6000 Sequencing System, the NextSeq 500 Sequencing System, the NextSeq 550 Sequencing System, or the NextSeq 550Dx Instrument, as well as Next-Generation Sequencing workflows and *in vitro* diagnostic procedures. The Illumina® COVIDSeq™ Test is only for use under the Food and Drug Administration's Emergency Use Authorization.

Summary and Explanation of the Assay

SARS-CoV-2 belongs to a large family of coronaviruses that lead to respiratory tract diseases in humans ranging from seasonal cold to severe infections, including Middle East Respiratory Syndrome (MERS) and Severe Acute Respiratory Syndrome (SARS).¹ SARS-CoV-2 leads to the COVID-19 disease, which is associated in the majority of infections with mild respiratory symptoms. However, for patients with underlying medical conditions and advanced age COVID-19 may lead to severe illness. ¹ The primary route of SARS-CoV-2 transmission between humans is via respiratory route, including droplets of saliva or discharge from infected patients. ¹ Confirmation of COVID-19 relies on detection of SARS-CoV-2 RNA from a patient during an ongoing, current infection. ²

The Illumina COVIDSeq Test is intended for detection of SARS-CoV-2 virus RNA under FDA Emergency Use Authorization and virus genome analysis for research use. Insight into the SARS-CoV-2 strain present in the sample enables tracking of virus strains. This test has been designed to sequence up to 3072 samples simultaneously using the NovaSeq 6000 system or up to 384 samples using the NextSeq 500/550 systems or NextSeq 550DX instrument to detect and sequence SARS-CoV-2 RNA and internal controls.

Principles of Procedure

The workflow consists of the following procedures: RNA extraction, cDNA synthesis, target amplification, library preparation, library pooling, sequencing, and analysis, which are outlined in more detail:

- ▶ **RNA Extraction**—RNA is extracted from decontaminated nasopharyngeal swabs using the Quick-DNA/RNA Viral MagBead Kit (Zymo Research, # R2141) or QIAamp Viral RNA Mini Kit (Qiagen, part # 52906).
- ▶ **cDNA Synthesis**—Generates DNA complementary to the RNA by reverse transcriptase with random hexamers.
- ▶ **Target Amplification**—The virus genome present in the sample is amplified using two separate PCR reactions that are then pooled together.
- ▶ **Library Preparation**—The pooled amplified fragments undergo tagmentation to further fragment and tag amplicons with adapter sequences. Post-tagmentation yield is normalized due to saturation of the bead-linked transposome by typical amplicon inputs. The adapter-tagged amplicons undergo a second round of PCR amplification using a PCR master mix and unique index adapters. After amplification, indexed libraries are pooled and cleaned using purification beads.
- ▶ **Quantification**—The pooled library product is quantified using a fluorescent dye with concentration determined by comparison to a DNA standard curve.
- ▶ **Sequencing**—Pooled libraries are clustered onto a flow cell, and then sequenced using sequencing by synthesis (SBS) chemistry on the NovaSeq 6000 Sequencing System using the NovaSeq Xp S4 and SP flow cells, NextSeq 500 System, NextSeq 550 System, and NextSeq 550Dx Instrument in RUO mode. SBS chemistry uses a reversible-terminator method to detect single, fluorescently labeled deoxynucleotide triphosphate (dNTP) bases as they are incorporated into growing DNA strands. During each sequencing cycle, a single dNTP is added to the nucleic acid chain. The dNTP label serves as a terminator for polymerization. After each dNTP incorporation, the fluorescent dye is imaged to identify the base, and then cleaved to allow incorporation of the next nucleotide. Four reversible terminator-bound dNTPs (A, G, T, and C) are present as single, separate molecules. As a result, natural competition minimizes incorporation bias. During the primary analysis, base calls are made directly from signal intensity measurements during each sequencing cycle, resulting in base by base sequencing. A quality score is assigned to each base call.
- ▶ **Analysis**—The Illumina DRAGEN COVIDSeq Test Pipeline analyzes sequencing results to detect the presence of SARS-CoV-2 RNA in each sample for diagnostic use under the FDA Emergency Use Authorization. Analysis can be performed locally using the Illumina DRAGEN COVIDSeq Test Pipeline or on BaseSpace Sequence Hub using the Illumina DRAGEN COVIDSeq Test app.
- ▶ For each result with at least 90 SARS-CoV-2 virus targets, the Illumina DRAGEN COVIDSeq Test Pipeline performs small variant calling and generates a consensus sequence in FASTA format for research use.

Warnings and Limitations of the Procedure

- ▶ This test has not been FDA cleared or approved.
- ▶ For *in vitro* diagnostic use only.
- ▶ For prescription use only.
- ▶ This test has been authorized by FDA under an EUA for use by laboratories certified under the Clinical Laboratory Improvement Amendments (CLIA) of 1988, 42 U.S.C. §263a, to perform high complexity tests.
- ▶ This test has been authorized only for the detection of nucleic acid from SARS-CoV-2, not for any other viruses or pathogens.
- ▶ This test 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 authorization is terminated or revoked sooner.

- ▶ Anterior nasal swabs, mid-turbinate swabs, oropharyngeal swabs, and bronchoalveolar lavage specimens are additional acceptable respiratory specimens that can be tested with the Illumina COVIDSeq Test; however, performance with these specimen types has not been determined.
- ▶ Use of the Illumina COVIDSeq Test is limited to personnel who have been trained in the procedures of molecular diagnostic assays including RT-PCR and the NovaSeq 6000 Sequencing System.
- ▶ Laboratories are required to report all positive results to the appropriate public health authorities.
- ▶ The instruments and assay procedures reduce the risk of contamination by amplification product. However, nucleic acid contamination from the positive controls or specimens must be controlled by good laboratory practices and careful adherence to the procedures specified in this package insert.
- ▶ Optimal performance of this test requires appropriate specimen collection, storage, and transport to the test site. For more information, see *Specimen Collection, Transport, and Storage on page 8*.
- ▶ Detection of SARS-CoV-2 RNA may be affected by sample collection methods, patient factors (eg, presence of symptoms), and/or stage of infection.
- ▶ False-negative results may arise from degradation of the viral RNA during shipping and storage.
- ▶ The impacts of vaccines, antiviral therapeutics, antibiotics, chemotherapeutic or immunosuppressant drugs have not been evaluated.
- ▶ As with any molecular test, mutations within the target regions of the Illumina COVIDSeq Test could affect primer binding resulting in failure to detect the presence of virus.
- ▶ 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 of a patient treatment and management or public health decision. Follow up testing should be performed according to the current CDC recommendations.
- ▶ Variant calls and consensus sequences performed by the Illumina DRAGEN COVIDSeq Test Pipeline are for information purposes only and should not be used for patient reporting.

Conditions of Authorization for the Laboratory

The Illumina COVIDSeq Test 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/emergency-situations-medical-devices/emergency-use-authorizations#covid19ivd>.

However, to assist clinical laboratories using the Illumina COVIDSeq Test, the relevant Conditions of Authorization are listed below:

- ▶ Authorized laboratories* using Illumina COVIDSeq Test will 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 Illumina COVIDSeq Test will use Illumina COVIDSeq Test as outlined in the Illumina COVIDSeq Test Instructions for Use. Deviations from the authorized procedures, including the authorized instruments, authorized extraction methods, authorized clinical specimen types, authorized control materials, authorized other ancillary reagents and authorized materials required to use Illumina COVIDSeq Test are not permitted.
- ▶ Authorized laboratories that receive Illumina COVIDSeq Test will notify the relevant public health authorities of their intent to run Illumina COVIDSeq Test prior to initiating testing.
- ▶ Authorized laboratories using Illumina COVIDSeq Test will have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.

- ▶ Authorized laboratories will collect information on the performance of Illumina COVIDSeq Test and report to DMD/OHT7-OIR/OPEQ/CDRH (via email: CDRH-EUA-Reporting@fda.hhs.gov) and Illumina Tech Support (via email: techsupport@illumina.com) any suspected occurrence of false positive or false negative results and significant deviations from the established performance characteristics of Illumina COVIDSeq Test of which they become aware.
 - ▶ All laboratory personnel using Illumina COVIDSeq Test must be appropriately trained in next generation sequencing and PCR techniques and use appropriate laboratory and personal protective equipment when handling this kit, and use Illumina COVIDSeq Test in accordance with the authorized labeling.
 - ▶ Illumina, authorized distributors, and authorized laboratories using Illumina COVIDSeq Test will ensure that any records associated with this EUA are maintained until otherwise notified by FDA. Such records will be made available to 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, to perform high complexity tests” as “authorized laboratories.”

Product Components

The Illumina COVIDSeq Test requires the following components:

- ▶ Illumina COVIDSeq Test (3072 Samples), part # 20043675
- ▶ 8 IDT for Illumina-PCR Indexes Sets 1-4 (384 Indexes), part # 20043137

Reagents

Reagents Provided



NOTE

Individual reagent tubes contain a label with the following language “For Research Use Only, not for use in diagnostic procedures”. These RUO labeled reagents have been authorized for use as IVD under the Emergency Use Authorization granted to this product.

Illumina COVIDSeq Test

Promptly store reagents at the indicated temperature to ensure proper performance.

Table 1 Illumina COVIDSeq Test Box 1 – 3072 Samples, Part # 20043645

Quantity	Label Volume	Reagent	Description	Storage
1	233 ml	ITB	Illumina Tune Beads	Room temperature

Table 2 Illumina COVIDSeq Test Box 2 – 3072 Samples, Part # 20043434

Quantity	Label Volume	Reagent	Description	Storage
1	55.6 ml	ST2 HT	Stop Tagment Buffer 2	Room temperature, post PCR environment

Table 3 Illumina COVIDSeqTest Box 3 – 3072 Samples, Part # 20043646

Quantity	Label Volume (ml)	Reagent	Description	Storage
2	6.15	EBLTS HT	Enrichment BLT	2°C to 8°C, post PCR environment
1	10	RSB HT	Resuspension Buffer	2°C to 8°C, post PCR environment

Table 4 Illumina COVIDSeq Test Box 4 – 3072 Samples, Part # 20043436

Quantity	Label Volume (ml)	Reagent	Description	Storage
1	114	ELB HT	Elution Buffer	2°C to 8°C, pre PCR environment
1	845	TWB HT	Tagmentation Wash Buffer HT	2°C to 8°C, post PCR environment

Table 5 Illumina COVIDSeq Test Box 5 – 3072 Samples, Part # 20043648

Quantity	Label Volume (ml)	Reagent	Description	Storage
1	45.1	EPH3 HT	Elution Prime Fragment 3HC Mix	-25°C to -15°C pre PCR environment
1	100.6	IPM HT	Illumina PCR Mix	-25°C to -15°C, pre PCR environment
1	78.9	EPM HT	Enhanced PCR Mix	-25°C to -15°C, pre PCR environment

Table 6 Illumina COVIDSeq Test Box 6 – 3072 Samples, Part # 20043647

Quantity	Label Volume (ml)	Reagent	Description	Storage
1	4.6	RVT HT	Reverse Transcriptase HT	-25°C to -15°C, pre PCR environment
1	41.1	FSM HT	First Strand Mix	-25°C to -15°C, pre PCR environment

Table 7 Illumina COVIDSeq Test Box 7 – 3072 Samples, Part # 20043439

Quantity	Label Volume (ml)	Reagent	Description	Storage
1	14.4	CPP1 HT	COVIDSeq Primer Pool 1	-25°C to -15°C, pre PCR environment
1	14.4	CPP2 HT	COVIDSeq Primer Pool 2	-25°C to -15°C, pre PCR environment
1	37.6	TB1 HT	Tagmentation Buffer 1 HT	-25°C to -15°C, post PCR environment

Table 8 Illumina COVIDSeq Positive Control HT, Part # 20043401

Quantity	Label Volume	Reagent	Description	Storage
1	100 µl	COVIDSeq Positive Control HT	COVIDSeq Positive control HT	-85°C to -65°C, post PCR environment

IDT for Illumina- PCR Indexes , Store at -25°C to -15°C

The Illumina COVIDSeq Test requires 8 IDT for Illumina PCR Indexes Sets 1–4 (384 Indexes) for a total 96 indexes, 96 sample index adapter plates.

Quantity	Description	Part Number
8	IDT for Illumina- PCR Indexes Set 1 (96 Indexes)	20043132
8	IDT for Illumina- PCR Indexes Set 2 (96 Indexes)	20043133
8	IDT for Illumina- PCR Indexes Set 3 (96 Indexes)	20043134
8	IDT for Illumina- PCR Indexes Set 4 (96 Indexes)	20043135

Reagents Not Provided

Reagents Required, Not Provided

- ▶ If using the QIAamp Viral RNA Mini Kit RNA extraction method:
 - ▶ 13 QIAamp Viral RNA Mini Kit, Qiagen, # 52906
 - ▶ QIAamp Viral RNA Mini Kit reagents. See *QIAamp Viral RNA Mini Handbook* (document # #HB-0354-006).
- ▶ If using the Quick- DNA/RNA Viral MagBead extraction method:
 - ▶ 8 Quick- DNA/RNA Viral MagBead, Zymo Research, # R2141
 - ▶ Quick- DNA/RNA Viral MagBead reagents. See *Quick-DNA/RNA Viral MagBead Instruction Manual*.
- ▶ Qubit dsDNA HS Assay Kit, Thermo Fisher Scientific, # Q32851 or Q32854
- ▶ If using the NovaSeq 6000 Sequencing System S4 flow cell, the following reagents for 3072 samples:
 - ▶ 2 NovaSeq 6000 Sequencing System S4 Reagent Kit (200 cycles), Illumina, # 20027466
 - ▶ 2 NovaSeq Xp 4-Lane Kit, Illumina, # 20021665
- ▶ If using the NovaSeq 6000 Sequencing System SP flow cell, the following NovaSeq 6000 Sequencing System reagents for 3072 samples:
 - ▶ 4 NovaSeq 6000 Sequencing System SP Reagent Kit (100 cycles), Illumina, # 20027464
 - ▶ 4 NovaSeq Xp 2-Lane Kit, Illumina, # 20021664
- ▶ If using the NextSeq 500/550 System or the NextSeq 550Dx instrument, the following reagents for 3072 samples:
 - ▶ 8 NextSeq 500/550 High Output Kit v2.5 (75 Cycles), Illumina, # 20024906
- ▶ 2 N NaOH
- ▶ 400 mM Tris-HCl, pH 8.0
- ▶ Nuclease-free water
- ▶ Ethanol, 100% (200 proof) of molecular biology grade, Sigma-Aldrich, # E7023



NOTE

Non-molecular biology grade ethanol can potentially negatively impact performance of the assay

Reagents Optional, Not Provided

- ▶ DNAZap
- ▶ RNaseZap

Storage and Handling

- 1 Room temperature is defined as 15°C to 30°C.
- 2 Do not allow multiple freeze-thaw cycles for CPC HT. If performing library prep multiple times, aliquot CPC HT into low-bind tubes, and then store at -85°C to -75°C.
- 3 Do not allow more than 8 freeze-thaw cycles for all reagents, excluding CPC HT.

- 4 Reagents are stable when stored as indicated until the specified expiration date on the kit labels. For storage conditions, see the Storage column in the tables in *Reagents Provided on page 1*. Do not use expired reagents.
- 5 Changes in the physical appearance of the reagents provided can indicate deterioration of the materials. If changes in the physical appearance occur (eg, obvious changes in reagent color or cloudiness apparent with microbial contamination), do not use the reagents.
- 6 Sequence libraries as soon as possible after pooling. Pooled libraries are stable for up to 30 days at -25°C to -15°C.

Equipment and Materials

Equipment and Materials Required, Not Provided

Equipment Required, Not Provided

Equipment	Supplier
10 µl single-channel pipettes	General lab supplier
20 µl single-channel pipettes	General lab supplier
200 µl single-channel pipettes	General lab supplier
1000 µl single-channel pipettes	General lab supplier
10 µl 8-channel pipettes	General lab supplier
20 µl 8-channel pipettes	General lab supplier
200 µl 8-channel pipettes	General lab supplier
1000 µl 8-channel pipettes	General lab supplier
20 µl 12-channel pipettes	General lab supplier
200 µl 12-channel pipettes	General lab supplier
10 ml serological pipettes	General lab supplier
25 ml serological pipettes	General lab supplier
50 ml serological pipettes	General lab supplier
BioShake iQ	QInstruments, part # 1808-0506
DRAGEN Bio-IT Platform or BaseSpace Sequence Hub	Illumina
Required equipment for one the following extraction methods:	<ul style="list-style-type: none"> • See <i>Quick-DNA/RNA Viral MagBead Instruction Manual</i> • See <i>QIAamp Viral RNA Mini Handbook (document # HB-0354-006)</i>
• Quick-DNA/RNA Viral MagBead equipment	
• QIAamp Viral RNA Mini Kit equipment	
Freezer, -25°C to -15°C	General lab supplier
Freezer, -85°C to -65°C	General lab supplier
Magnetic Stand-96	Thermo Fisher Scientific, catalog # AM10027
One of the following magnetic stands:	
• Dynabeads MPC-S (Magnetic Particle Concentrator)	• Thermo Fisher Scientific, catalog #A13346
• MagnaRack Magnetic Separation Rack	• Thermo Fisher Scientific, catalog # CS15000
Microcentrifuge	General lab supplier
Microplate Centrifuge	General lab supplier

Equipment	Supplier
One of the following sequencing systems: <ul style="list-style-type: none"> • NextSeq 500 • NextSeq 550 • NextSeq 550Dx • NovaSeq 6000 	Illumina
NovaSeq Xp Flow Cell Dock	Illumina, # 20021663
Pipette Aid	General lab supplier
Quibit Fluorometer 3.0	Thermo Fisher, catalog # Q33216, Q33217, or Q33218
Refrigerator, 2°C to 8°C	General lab supplier
One of the following thermal cyclers: <ul style="list-style-type: none"> • C1000 Touch™ Thermal Cycler with 96-Well Fast Reaction Module • C1000 Touch™ Thermal Cycler with 96-Deep Well Reaction Module 	<ul style="list-style-type: none"> • Bio-Rad, part # 1851196 • Bio-Rad, part # 1851197
Sealing wedge or roller	General lab supplier
Vortexer	General lab supplier

Materials Required, Not Provided

Consumable	Supplier
10 µl pipette tips	General lab supplier
20 µl pipette tips	General lab supplier
200 µl pipette tips	General lab supplier
200 µl pipette tips	General lab supplier
1000 µl pipette tips	General lab supplier
Hard-Shell 96-Well PCR Plates	Bio-Rad, catalog # HSP-9601 or equivalent
96 deep well plate, 2000 µl	Eppendorf, catalog # 951033707
1.7 ml LoBind microcentrifuge tubes	Eppendorf, catalog # 022431021
5 ml LoBind microcentrifuge tube	Eppendorf, catalog # 0030122348
15 ml tubes	General lab supplier
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
RNase/DNase-free Disposable Pipetting Reservoirs	VWR, part # 89094-658
Qubit dsDNA HS Assay Kit	One of the following, depending on kit size: <ul style="list-style-type: none"> • ThermoFisher Scientific, part # Q32851 • ThermoFisher Scientific, part # Q32854
Qubit Assay Tubes	ThermoFisher Scientific, catalog # Q32856

Specimen Collection, Transport, and Storage



CAUTION

Handle all specimens as infectious agents.

- 1 Nasopharyngeal swab samples collected in viral transport tubes are acceptable. In addition, anterior nasal swabs, mid-turbinate swabs, oropharyngeal swabs, and bronchoalveolar lavage specimens are acceptable respiratory specimens that can be tested with the Illumina COVIDSeq Test. However, performance with these specimen types has not been determined.

- 2 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.
- 3 Transportation of patient samples must comply with all applicable governing regulations for the transport of etiologic agents.

**CAUTION**

Exceeding the specified storage times can negatively impact test results.

Warnings and Precautions

- ▶ This assay contains potentially hazardous chemicals. For health and safety information, review Safety Data Sheets (SDS) available on the Illumina 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 container. Dispose 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.
- ▶ For environmental, health, and safety information, see the safety data sheets (SDS) at support.illumina.com/sds.html.

Procedural Notes

Avoiding Contamination

- ▶ Use proper laboratory practices to prevent nucleases and PCR products contamination. Nuclease and PCR product contamination can cause inaccurate and unreliable results.
- ▶ Perform library preparation in a RNase/DNase-free environment. Thoroughly decontaminate work areas with a RNase/DNase-inhibiting solution, such as RNaseZap and DNazap.
- ▶ Use fresh tips and fresh consumable labware between samples and dispensing reagents.
- ▶ Use aerosol-resistant tips to reduce the risk of carry-over and sample-to-sample cross-contamination.

- ▶ Due to the potential for 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.

Quality Control

Control material with known performance characteristics may be evaluated to detect differences in processing and technical procedures in the laboratory. Include a no template control (NTC) and positive control with every sample prep batch. The Illumina COVIDSeq Test includes COVIDSeq Positive Control HT to use as a positive control. COVIDSeq Positive Control HT consists of ssRNA fragments that provide coverage of greater than 99.9% of the SARS-CoV-2 viral genome bases. The Illumina COVIDSeq Test also includes internal human target controls for each sample in the assay as a process control to prepare libraries for sequencing.

The following external controls are used in the Illumina COVIDSeq Test:

- ▶ **ELB HT (Elution Buffer HT)**, Illumina part # 20043436—No Template Control (NTC) used in the Illumina COVIDSeq Test for 94 unique test samples and to dilute the COVIDSeq Positive Control HT at 5000 copies per ml. One control is required for 94 samples.

The ELB HT monitors the following actions:

- ▶ Cross-contamination during RNA extraction and downstream library prep steps.
- ▶ Reagent integrity.
- ▶ **COVIDSeq Positive Control HT**, Illumina part # 20043401—SARS-CoV-2 positive control used in the Illumina COVIDSeq Test for 94 samples. One control is required for 94 samples.

COVIDSeq Positive Control HT monitors the following actions:

- ▶ Positive control used to detect SARS-CoV-2.
- ▶ Reagent integrity.

The following internal controls are used in the Illumina COVIDSeq Test:

- ▶ **CCP1 HT (COVIDSeq Primer Pool 1 HT)** and **CCP2 HT (COVIDSeq Primer Pool 2 HT)** Illumina, part # 20043439—A total of two pools that include SARS-CoV-2 specific primers and a set of primer pairs targeting human transcripts. The targeted human transcripts are commonly prevalent in nasopharyngeal swabs, oropharyngeal swabs, nasal swab, and mid-turbinate specimens. Therefore, these human target primers act as internal process control for each sample that will be used in the Illumina COVIDSeq Test.

CCP1 and CCP2 monitor the following actions:

- ▶ Successful completion of each step from extraction through DNA tagmentation, library prep, and sequencing to validate negative samples when the SARS-CoV-2 virus is not detected and "PASS" is displayed as the internal control result.



NOTE

Illumina requires using one NTC and one positive control per 96-well plate. For library preparation of each sample, the internal process control is included in the Illumina COVIDSeq Test.

Instructions for Use

Tips and Techniques

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Sealing and Unsealing the Plate

- ▶ Always seal the 96-well plate before the following steps in the protocol:
 - ▶ Shaking steps

- ▶ Vortexing steps
- ▶ Centrifuge steps
- ▶ Thermal cycling steps
- ▶ To seal the plate, apply the adhesive cover to the plate and then seal with a wedge or rubber roller.
- ▶ Make sure the edges and wells are completely sealed to reduce the risk of cross-contamination and evaporation.
- ▶ Microseal 'B' adhesive seals are effective at -40°C to 110°C, and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' for shaking, centrifuging, and long-term storage.
- ▶ Before unsealing:
 - ▶ Briefly centrifuge the 96-well plate at 1000 × g for 1 minute. For bead steps, centrifuge at 500 × g for 1 minute.
 - ▶ Place the plate on a flat surface before slowly removing the seal.

Plate Transfers

- ▶ When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.
- ▶ If beads are aspirated into the pipette tips, dispense back to the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

Centrifugation

- ▶ Centrifuge as needed at any step in the procedure to consolidate liquid or beads in the bottom of the well, and to prevent sample loss.

Handling Beads

- ▶ Pipette bead suspension slowly to prevent splashing and bubbles.
- ▶ When mixing, mix thoroughly.
- ▶ To avoid sample loss, confirm that no beads remain in pipette tips after resuspension and mixing steps.
- ▶ When washing beads:
 - ▶ Use the appropriate magnet for the plate.
 - ▶ Dispense liquid so that beads on the side of the wells are wetted.
 - ▶ Keep the plate on the magnet until the instructions specify to remove it.
 - ▶ Do not agitate the plate while on the magnetic stand. Do not disturb the bead pellet.

Extract RNA

This step extracts RNA from decontaminated viral transport medium tubes. You can extract RNA using the Quick-DNA/RNA Viral MagBead, Zymo Research, part # R2141 or the QIAamp Viral RNA Mini Kit, Qiagen, part # 52906. Follow the procedure corresponding to your extraction method.

Consumables

- ▶ ELB HT (Elution Buffer HT)
- ▶ CPC HT (COVIDSeq Positive Control HT)
- ▶ 1.7 ml LoBind tubes
- ▶ 5 ml LoBind tubes
- ▶ 15 ml tubes
- ▶ **[Quick-DNA/RNA Viral MagBead]** 2000 µl 96 deep well plate

About Reagents

- ▶ Aliquot CPC HT into low-bind tubes. Store at -85°C to -65°C
- ▶ Vortex before each use

Preparation

- 1 Prepare the following consumables:

Reagent	Storage	Instructions
ELB HT	2°C to 8°C	Thaw at room temperature, and then invert to mix. Keep on ice until use.
CPC HT	-85°C to -65°C	Dilute to 5 copies per μl using the following instructions. Keep diluted positive control on ice.

- 2 Dilute CPC HT as follows.

- a Label a 1.7 ml tube Dilution 1.
- b Add the following volumes to the tube ***in the order listed***.
 - ▶ CPC HT (5 μl)
 - ▶ ELB HT (495 μl)
 These volumes produce 10000 copies per μl .
- c Pulse vortex to mix.

- 3 Dilute CPC HT a second time as follows.

- a Label a 1.7 ml tube Dilution 2.
- b Add the following volumes to the tube ***in the order listed***.
 - ▶ Dilution 1 (5 μl)
 - ▶ ELB HT (495 μl)
 These volumes produce 100 copies per μl .
- c Pulse vortex to mix.

- 4 Dilute CPC HT a third time as follows.

- a Label a 15 ml tube Dilution 3.
- b Add the following volumes to the tube ***in the order listed***.
 - ▶ Dilution 2 (200 μl)
 - ▶ ELB HT (3.8 ml)
 These volumes produce 5 copies per μl .
- c Pulse vortex to mix.

Quick-DNA/RNA Viral MagBead Procedure

- 1 For each sample, add 400 μl patient sample to a new deep-well plate. For every 94 samples, include one tube of dilution 3 CPC HT (positive control) and ELB HT (no template control).
- 2 To extract RNA, use the Quick-DNA/RNA Viral MagBead. For information, see *Quick-DNA/RNA Viral MagBead Instruction Manual* available from Zymo Research.
Use the following protocol options:
 - ▶ After adding 20 μl MagBinding Beads, shake at 1500 rpm for 10 minutes.
 - ▶ Mix well by pipetting up and down ten times.

QIAamp Viral RNA Mini Kit Procedure

- 1 For each sample, add 140 μl patient sample to new 1.7 ml microcentrifuge tube. For every 94 samples, include one tube of dilution 3 CPC HT (positive control) and ELB HT (no template control).

- To extract RNA, use the QIAamp Viral RNA Mini Kit. For information, see *QIAamp Viral RNA Mini Handbook* (document # HB-0354-006) available on the QIAGEN website.

Use the following protocol options:

- ▶ Purify viral RNA using the spin protocol.
- ▶ Incubate elution for at least 1 minute.
- ▶ Elute in 30 µl Buffer AVE instead of 60 µl.

Anneal RNA

During this process the extracted RNA is annealed using random hexamers to prepare for cDNA synthesis.

Consumables

- ▶ EPH3 HT (Elution Prime Fragment 3HC Mix HT)
- ▶ 96-well PCR Plate
- ▶ Microseal 'B' adhesive seals

About Reagents

- ▶ Vortex before each use

Preparation

- Prepare the following consumables:

Reagent	Storage	Instructions
EPH3 HT	-25°C to -15°C	Thaw at room temperature, and then invert to mix.

- Save the following COVIDSeq Anneal program on the thermal cycler:
 - ▶ Choose the preheat lid option
 - ▶ Set the reaction volume to 17 µl
 - ▶ 65°C for 3 minutes
 - ▶ Hold at 4°C

Procedure

- Label new PCR plate CDNA1.
- Add 8.5 µl EPH3 HT to each well.
- Add 8.5 µl eluted sample to each well.
- Seal and shake at 1600 rpm for 1 minute.
- Centrifuge at 1000 × g for 1 minute.
- Place on the preprogrammed thermal cycler and run the COVIDSeq Anneal program.

Synthesize First Strand cDNA

This step reverse transcribes the RNA fragments primed with random hexamers into first strand cDNA using reverse transcriptase.

Consumables

- ▶ FSM HT (First Strand Mix HT)
- ▶ RVT HT (Reverse Transcriptase HT)
- ▶ 1.7 ml tubes (1 per 96-well sample plate)
- ▶ Microseal 'B' adhesive seal

Preparation

- 1 Prepare the following consumables:

Reagent	Storage	Instructions
FSM HT	-25°C to -15°C	Thaw and bring to room temperature. Invert to mix, and then keep on ice.
RVT HT	-25°C to -15°C	Invert to mix before use. Keep on ice.

- 2 Save the following COVIDSeq FSS program on the thermal cycler:

- ▶ Choose the preheat lid option
- ▶ Set the reaction volume to 25 µl
- ▶ 25°C for 5 minutes
- ▶ 50°C for 10 minutes
- ▶ 80°C for 5 minutes
- ▶ Hold at 4°C

Procedure

- 1 In a 1.7 ml tube, combine the following volumes to prepare First Strand cDNA Master Mix. Multiply each volume by the number of samples.
 - ▶ FSM HT (9 µl)
 - ▶ RVT HT (1 µl)
 Reagent overage is included to account for small pipetting errors.
- 2 Add 8 µl master mix to each well of the CDNA1 plate.
- 3 Seal and shake at 1600 rpm for 1 minute.
- 4 Centrifuge at 1000 × g for 1 minute.
- 5 Place on the preprogrammed thermal cycler and run the COVIDSeq FSS program.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

Amplify cDNA

This step uses two separate PCR reactions to amplify cDNA.

Consumables

- ▶ IPM HT (Illumina PCR Mix HT)
- ▶ CPP1 HT (COVIDSeq Primer Pool 1 HT)
- ▶ CPP2 HT (COVIDSeq Primer Pool 2 HT)
- ▶ Nuclease-free water
- ▶ 15 ml tube (2 for four 96-well sample plates)
- ▶ 96-well PCR plates (3)
- ▶ Microseal 'B' adhesive seal

Preparation

- 1 Prepare the following consumables:

Reagent	Storage	Instructions
CPP1 HT	-25°C to -15°C	Thaw at room temperature. Keep on ice until use.
CPP2 HT	-25°C to -15°C	Thaw at room temperature. Keep on ice until use.
IPM HT	-25°C to -15°C	Thaw at room temperature, and then invert to mix. Keep on ice until use.

- 2 Save the following COVIDSeq PCR program on the thermal cycler:
 - ▶ Choose the preheat lid option
 - ▶ Set the reaction volume to 25 µl
 - ▶ 98°C for 3 minutes
 - ▶ 35 cycles of:
 - ▶ 98°C for 15 seconds
 - ▶ 65°C for 5 minutes
 - ▶ Hold at 4°C

Procedure

- 1 Label two new PCR plates COV1 and COV2.
The plates represent two separate PCR reactions on each sample and control in the CDNA1 plate.
- 2 In a 15 ml tube, combine the following volumes to prepare COVIDSeq PCR 1 Master Mix and COVIDSeq PCR 2 Master Mix. Multiply each volume by the number of samples.

Reagent	COVIDSeq PCR 1 Master Mix (µl)	COVIDSeq PCR 2 Master Mix (µl)
IPM HT	15	15
CPP1 HT	4.3	N/A
CPP2 HT	N/A	4.3
Nuclease-free water	4.7	4.7

Reagent overage is included to account for small pipetting errors.

- 3 Add 20 µl COVIDSeq PCR 1 Master Mix to each well of the COV1 plate corresponding to each well of the CDNA1 plate.
- 4 Add 5 µl first strand cDNA synthesis from each well of the CDNA1 plate to the corresponding well of the COV1 plate.
- 5 Add 20 µl COVIDSeq PCR 2 Master Mix to each well of the COV2 plate corresponding to each well of the CDNA1 plate.
- 6 Add 5 µl first strand cDNA synthesis from each well of the CDNA1 plate to the corresponding well of the COV2 plate.
- 7 Seal and shake at 1600 rpm for 1 minute.
- 8 Centrifuge at 1000 x g for 1 minute.
- 9 Place in the preprogrammed thermal cycler and run the COVIDSeq PCR program.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 3 days.

Tagment PCR Amplicons

This step uses EBLTS HT to tagment PCR amplicons, which is a process that fragments and tags the PCR amplicons with adapter sequences.

Consumables

- ▶ EBLTS HT (Enrichment BLT HT)
- ▶ TB1 HT (Tagmentation Buffer 1 HT)
- ▶ Nuclease-free water
- ▶ 1.7 ml tube
- ▶ 15 ml tube (1 per four 96-well sample plates)
- ▶ Microseal 'B' adhesive seal

About Reagents

- ▶ Store EBLTS HT upright at temperatures above 2°C. Make sure beads are always submerged in the buffer.
- ▶ If beads are adhered to the side or top of the 96-well plate, centrifuge at 500 × g for 1 minute, and then pipette to resuspend.

Preparation

- 1 Prepare the following consumables:

Reagent	Storage	Instructions
EBLTS HT	2°C to 8°C	Bring to room temperature. Vortex thoroughly before use.
TB1 HT	-25°C to -15°C	Bring to room temperature. Vortex thoroughly before use.

- 2 If COV1 and COV2 plates were stored frozen, prepare as follows.
 - a Thaw at room temperature.
 - b Check seals, and then shake at 1600 rpm for 1 minute.
 - c Centrifuge at 1000 × g for 1 minute.
- 3 Save the following COVIDSeq TAG program on the thermal cycler:
 - ▶ Choose the preheat lid option
 - ▶ Set the reaction volume to 50 µl
 - ▶ 55°C for 5 minutes
 - ▶ Hold at 10°C

Procedure

- 1 Label a new PCR plate TAG1.
- 2 Combine COV1 and COV2 as follows.
 - a Transfer 10 µl from each well of the COV1 plate to the corresponding well of the TAG1 plate.
 - b Transfer 10 µl from each well of the COV2 plate to each well of the TAG1 plate containing COV1.
- 3 In a 15 ml tube, combine the following volumes to prepare Tagmentation Master Mix. Multiply each volume by the number of samples.
 - ▶ TB1 HT (12 µl)
 - ▶ EBLTS HT (4 µl)
 - ▶ Nuclease-free water (20 µl)
- 4 Add 30 µl master mix to each well in TAG1 plate.
- 5 Seal and shake at 1600 rpm for 1 minute.
- 6 Place on the preprogrammed thermal cycler and run the COVIDSeq TAG program.

Post Tagmentation Clean Up

This step washes the adapter-tagged amplicons before PCR amplification.

Consumables

- ▶ ST2 HT (Stop Tagment Buffer 2 HT)
- ▶ TWB HT (Tagmentation Wash Buffer HT)
- ▶ Microseal 'B' adhesive seal

About Reagents

- ▶ Dispense ST2 HT and TWB HT slowly to minimize foaming.

- ▶ Dispense TWB HT directly onto beads.

Preparation

- 1 Prepare the following consumables:

Reagent	Storage	Instructions
ST2 HT	Room temperature	Vortex before use.
TWB HT	2°C to 8°C	Vortex before use.

Procedure

- 1 Centrifuge the TAG1 plate at 500 x g for 1 minute.
- 2 Add 10 µl ST2 HT to each well of the TAG1 plate.
- 3 Seal and shake at 1600 rpm for 1 minute.
- 4 Incubate at room temperature for 5 minutes.
- 5 Centrifuge at 500 x g for 1 minute.
- 6 Place on the magnetic stand and wait until the liquid is clear (~3 minutes).
- 7 Inspect for bubbles on the seal. If present, centrifuge at 500 x g for 1 minute, and then place on the magnetic stand (~3 minutes).
- 8 Remove and discard all supernatant.
- 9 Wash beads as follows.
 - a Remove from the magnetic stand.
 - b Add 100 µl TWB HT to each well.
 - c Seal and shake at 1600 rpm for 1 minute.
 - d Centrifuge 500 x g for 1 minute.
 - e Place on the magnetic stand and wait until the liquid is clear (~3 minutes).
 - f For first wash only, remove and discard all supernatant from each well.
- 10 Wash beads a **second** time.
Leave supernatant in plate for second wash to prevent beads from overdrying.

Amplify Tagmented Amplicons

This step amplifies the tagmented amplicons using a PCR program. The PCR step adds prepared 10 base pair Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for sequencing cluster generation.

Consumables

- ▶ EPM HT (Enhanced PCR Mix HT)
- ▶ Index adapters (IDT for Illumina-PCR Indexes Set 1, 2, 3, 4)
- ▶ Nuclease-free water
- ▶ 15 ml tubes (1 per two 96-well sample plates)
- ▶ 96-well PCR plate

About Reagents

- ▶ Index adapter plates
 - ▶ Do not add samples to the index plate wells.
 - ▶ Index plate wells cannot be reused.

Preparation

- 1 Prepare the following consumables:

Reagent	Storage	Instructions
EPM HT	-25°C to -15°C	Invert to mix. Keep on ice until use.
Index adapters	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge at 1000 × g for 1 minute.

- 2 Open each prepared index adapter plate seal as follows. Use a new PCR plate for each different index set.
 - a Align a new 96-well PCR plate above the index adapter plate, and then press down to puncture the foil seal.
 - b Discard the PCR plate.
- 3 Save the following COVIDSeq TAG PCR program on the thermal cycler:
 - ▶ Choose the preheat lid option and set to 100°C
 - ▶ Set the reaction volume to 50 µl
 - ▶ 72°C for 3 minutes
 - ▶ 98°C for 3 minutes
 - ▶ 7 cycles of:
 - ▶ 98°C for 20 seconds
 - ▶ 60°C for 30 seconds
 - ▶ 72°C for 1 minute
 - ▶ 72°C for 3 minutes
 - ▶ Hold at 10°C

Procedure

- 1 In a 15 ml tube, combine the following volumes to prepare PCR Master Mix. Multiply each volume by the number of samples.
 - ▶ EPM HT (24 µl)
 - ▶ Nuclease-free water (24 µl)
- 2 Vortex PCR Master Mix to mix.
- 3 Keep the TAG1 plate on magnetic stand and remove TWB HT.
- 4 Use a 20 µl pipette to remove any remaining TWB HT.
- 5 Remove the TAG1 plate from the magnetic stand.
- 6 Add 40 µl PCR Master Mix to each well.
- 7 Add 10 µl index adapters to each well of the PCR plate.
- 8 Seal and shake at 1600 rpm for 1 minute.
- 9 If liquid is visible on the seal, centrifuge at 500 × g for 1 minute.
- 10 Inspect to make sure beads are resuspended. To resuspend, set your pipette to 35 µl with the plunger down, and then slowly pipette to mix.
- 11 Place on the preprogrammed thermal cycler and run the COVIDSeq TAG PCR program.

Pool and Clean Up Libraries

This step combines libraries from each 96-well sample plate into one 1.7 ml tube. Libraries of optimal size are then bound to magnetic beads, and fragments that are too small or large are washed away.

Consumables

- ▶ ITB (Illumina Tune Beads)
- ▶ RSB HT (Resuspension Buffer HT)
- ▶ Freshly prepared 80% ethanol (EtOH)
- ▶ 1.7 ml tube (2 per 96-well sample plate)
- ▶ PCR 8-tube strip

About Reagents

- ▶ ITB
 - ▶ Vortex before each use.
 - ▶ Vortex frequently to make sure that beads are evenly distributed.
 - ▶ Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

- 1 Prepare the following consumables:

Reagent	Storage	Instructions
ITB	Room temperature	Vortex thoroughly to mix.
RSB HT	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.

- 2 Prepare 80% EtOH from absolute EtOH.

Procedure

- 1 Centrifuge at 500 × g for 1 minute.
- 2 Place on the magnetic stand and wait until the liquid is clear (~3 minutes).
- 3 To pool libraries, do as follows. Repeat the steps for each additional sample plate.
 - a Use a 20 µl eight-channel pipette to transfer 5 µl library from each well of the PCR plate to a PCR 8-tube strip. Change tips after each column.
These volumes result in 60 µl pooled library per row.
 - b Label a new 1.7 ml tube Pooled ITB.
 - c Transfer 55 µl pooled library from each well of the PCR 8-tube strip into the Pooled ITB tube.
For each sample plate, these volumes results in 440 µl pools of pooled libraries.

If processing 3072 samples, these steps result in 32 Pooled ITB tubes.

- 4 Vortex the Pooled ITB tubes to mix, and then centrifuge briefly.
- 5 Vortex ITB to resuspend.
- 6 Add ITB using the resulting volume of Pooled ITB tube volume multiplied by 0.9.
For example, for 96 samples, add 396 µl ITB to each tube.
- 7 Vortex to mix.
- 8 Incubate at room temperature for 5 minutes.
- 9 Centrifuge briefly.
- 10 Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
- 11 Remove and discard all supernatant.
- 12 Wash beads as follows.
 - a Keep on the magnetic stand and add 1000 µl fresh 80% EtOH to each tube.
 - b Wait 30 seconds.
 - c Remove and discard all supernatant.
- 13 Wash beads a **second** time.
- 14 Use a 20 µl pipette to remove all residual EtOH.
- 15 Add 55 µl RSB HT.
- 16 Vortex to mix, and then centrifuge briefly.
- 17 Incubate at room temperature for 2 minutes.
- 18 Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
- 19 Transfer 50 µl supernatant from each Pooled ITB tube to a new microcentrifuge tube.

SAFE STOPPING POINT

If you are stopping, cap the tubes and store at -25°C to -15°C for up to 30 days.

Quantify and Normalize Libraries

- 1 Analyze 2 µl library pool using a Qubit dsDNA HS Assay kit.
If libraries are outside the standard range, dilute to 1:10 concentration, and analyze again.
- 2 Calculate the molarity value using the following formula.
 - ▶ Use 400 bp as the average library size.

$$\frac{\text{Library concentration ng/}\mu\text{l}}{660 \frac{\text{g}}{\text{mol}} \times \text{average library size (bp)}} \times 10^6 = \text{Molarity (nM)}$$

- 3 Dilute each library pool to a minimum of 30 µl at a normalized concentration 4 nM using RSB HT.

Pool and Dilute Libraries

This step pools and dilutes libraries to the starting concentration for your sequencing system. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration.

- 1 For each set of 384 samples, combine 25 µl of each normalized pool containing index adapter set 1, 2, 3, 4 in a new microcentrifuge tube. Do not combine pools with the same index adapter set.
This step produces a final pool of 384 samples diluted to a starting concentration of 4 nM. For each sequencing system, the following are the number of samples required per flow cell.
 - ▶ NextSeq 500/550 or 550Dx HO flow cell: 384 samples per flow cell.
 - ▶ NovaSeq 6000 system SP flow cell: 384 samples per lane and 768 total samples per flow cell.
 - ▶ NovaSeq 6000 system S4 flow cell: 384 samples per lane and 1536 total samples per flow cell.
- 2 Follow the denature and dilute instructions for your system to dilute to the final loading concentration.
 - ▶ For the NextSeq 500/550 Sequencing System and NextSeq 550Dx Sequencing System, see the *NextSeq System Denature and Dilute Libraries Guide* (document # 15048776).
 - ▶ For the NovaSeq 6000 Sequencing System, see the *NovaSeq 6000 Denature and Dilute Libraries Guide* (document # 1000000106351).
- 3 Use the following loading concentrations for your system.

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)
NextSeq 500/550 or 550Dx HO flow cell	4	1.4
NovaSeq 6000 SP Flow Cell	4	100
NovaSeq 6000 S4 Flow Cell	4	100

Adjustments to final loading concentration should follow the denature and dilute instructions for your sequencing system.

Prepare for Sequencing**Consumables**

- ▶ If using the NovaSeq 6000 Sequencing System S4 flow cell:
 - ▶ 2 NovaSeq 6000 Sequencing System S4 Reagent Kit (200 cycles), Illumina, # 20027466
 - ▶ 2 NovaSeq Xp 4-Lane Kit, Illumina, # 20021665
- ▶ If using the NovaSeq 6000 Sequencing System SP flow cell:
 - ▶ 4 NovaSeq 6000 Sequencing System SP Reagent Kit (100 cycles), Illumina, # 20027464
 - ▶ 4 NovaSeq Xp 2-Lane Kit, Illumina, # 20021664
- ▶ If using the NextSeq 500/550 System or NextSeq 550Dx Instrument:

- 8 NextSeq 500/550 High Output Kit v2.5 (75 Cycles), Illumina, # 20024906

Sample Sheet Requirements

The Illumina DRAGEN COVIDSeq Test Pipeline requires a sample sheet for each run analysis. Use the `samplesheet.csv` file for your sequencing system included in the installer packager or available on the Illumina COVIDSeq Test support site as a template to create the sample sheet.

Make sure your sample sheet meets the following requirements.

- 1 Save the sample sheet with the name `SampleSheet.csv` in the sequencing run folder.
- 2 In Settings, enter the following value for the `AdapterRead1` parameter.

CTGTCTCTTATACACATCT

- 3 In the Data section, enter the following required parameters.

Make sure that there no empty rows between samples.

Field	Description	Requirements
Sample_ID	The ID used to identify the samples in the test reports and included in the output file names.	Sample IDs are not case-sensitive. Make sure Sample IDs contain the following: <ul style="list-style-type: none"> • Unique for the run. • ≤ 100 characters with no spaces. • Alphanumeric characters, underscores, and dashes only. An alphanumeric character must be added before and after an underscore or dash.
Index_ID	The IDT for Illumina-PCR Indexes index name associated with the sample.	See <i>Illumina Adapter Sequences (document # 1000000002694)</i> for index names and additional information. The name must be unique for each flow cell lane. If the Index_ID is not specified, the Index Set field is derived from Index and Index2. If specifying all three, the index names and associated sequences must match.
Index	IDT for Illumina-PCR Indexes i7 index sample sheet bases	See <i>Illumina Adapter Sequences (document # 1000000002694)</i> for sample sheet bases for your sequencing system and additional information. If Index_ID is specified, Index is not required.
Index2	IDT for Illumina-PCR Indexes i5 index sample sheet bases.	See <i>Illumina Adapter Sequences (document # 1000000002694)</i> for sample sheet bases for your sequencing system and additional information. If Index_ID is specified, Index2 is not required.
Lane	The flow cell lane for the sample.	If using the NovaSeq 6000 System, enter one of the following values: 1, 2, 3, or 4. If using the NextSeq 500/550 or NextSeq 500Dx, this field is not included.
Sample_Type	The sample type for each sample.	Enter one of the following case-sensitive values: PatientSample, NTC, PositiveControl. If using the NovaSeq 6000 System, there must be one NTC sample and one PostiveControl sample for each Index Set/Lane combination in the sample sheet. If using the NextSeq 500/550 or 550Dx, there must be one NTC sample and one PostiveControl sample for each Index Set combination in the sample sheet

- 4 **[Optional]** Enter any additional data parameters, such as `Sample_Name`.

Set Up Sequencing Run

- 1 If using the NovaSeq 6000 system, refer to the *NovaSeq 6000 Sequencing System Guide (document # 1000000019358)* for sequencing instructions.
 - Use v1.6 of the NovaSeq Control Software (NVCS).

- ▶ If using the Illumina DRAGEN COVIDSeq Test BaseSpace Sequence Hub app, select Run Monitoring and Storage as the Configuration option.
 - ▶ Use the following number of cycles and index lengths:
 - ▶ **Read 1**—Enter 36 as the value.
 - ▶ **Index 1** and **Index 2**—Enter 10 as the value.
 - ▶ **Read 2**—Enter 0 as the value.
- 2 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).
- ▶ Use v4.0 of the NextSeq Control Software (NCS).
 - ▶ If using the NextSeq 550Dx, use RUO mode.
 - ▶ Set up your sequencing run in manual mode.
 - ▶ If using the Illumina DRAGEN COVIDSeq Test BaseSpace Sequence Hub app, select Run Monitoring and Storage as the Configuration option.
 - ▶ Enter Single-Read as the Read Type.
 - ▶ Use the following number of cycles and index lengths:
 - ▶ **Read 1**—Enter 36 as the value.
 - ▶ **Index 1** and **Index 2**—Enter 10 as the value.

After sequencing is complete, perform analysis on your system using the Illumina DRAGEN COVIDSeq Test Pipeline or in BaseSpace Sequence Hub using the Illumina DRAGEN COVIDSeq Test. For information on performing analysis locally, see the *Illumina DRAGEN COVIDSeq Test Pipeline Software Guide* (document # 1000000128119). For information on performing analysis on BaseSpace Sequence Hub, see *Illumina DRAGEN COVIDSeq Test App Guide* (document # 1000000129048).

Interpretation of Results

SARS-CoV-2 Detected in Sample ID	Internal Control Sample ID	NTC	Positive Control	Sample Result	Interpretation	Action
+	Pass or N/A	Pass	Pass	SARS-CoV-2 Detected	Positive for SARS-CoV-2 for the Sample ID.	Report results to physician, patient, and appropriate public health authorities.
-	Pass	Pass	Pass	SARS-CoV-2 Not Detected	Negative for SARS-CoV-2 for the Sample ID.	Report results to physician, patient, and appropriate public health authorities.
-	Fail	Pass	Pass	Invalid	Invalid for the Sample ID.	Quality control for the Sample ID is FAIL. Re-extract sample and repeat Illumina COVIDSeq Test for the sample
+ or -	Pass or Fail	Fail	Pass	Invalid*	All samples in the Index Set are Invalid.	Quality control for the Index set is FAIL. Repeat Illumina COVIDSeq Test for all Invalid samples of failed Index Set.
+ or -	Pass or Fail	Pass	Fail	Invalid*	All samples in the Index Set are Invalid.	Quality control for the Index set is FAIL. Repeat Illumina COVIDSeq Test for all Invalid samples of failed Index Set.
+ or -	Pass or Fail	Fail	Fail	Invalid*	All samples in the Index Set are Invalid.	Quality control for the Index set is FAIL. Repeat Illumina COVIDSeq Test for all Invalid samples of failed Index Set.

*If the positive control fails for a given index set, all patient samples associated with that index set are reported as invalid.

Performance Characteristics

The following data outlined in the clinical performance and analytical performance sections were generated by using the protocols and materials outlined in the Instructions for Use starting with nasopharyngeal (NP) swab samples. All sequencing data for this section were generated on a NovaSeq 6000 Sequencing System.

Analytical Sensitivity

The analytical sensitivity (Limit of Detection (LOD)) of the Illumina COVIDSeq Test using the Zymo extraction method was determined by serial dilution of heat inactivated SARS-CoV-2 virus with known concentration (ATCC VR-1986HK) into pooled negative clinical matrix (nasopharyngeal swab specimen) to 1000 copies per ml, 750 copies per ml, 500 copies per ml, and 250 copies per ml. For each serial dilution, 22–24 extraction replicates were used for two library preparations and evaluated using the Illumina COVIDSeq Test. The LOD using Zymo extraction was determined to be 500 copies per ml.

Sequencing System	ATCC Heat Inactivated Virus (copies per ml)	Replicate Breakdown				Detection Rate		
		Zymo Extractions	Library Preparations	Detected	Total	Percent Detected	Lower CI	Upper CI
NextSeq 550 (High output)	250	22	2	38	44	86.4%	73.3%	93.6%
	500	24	2	48	48	100.0%	92.6%	100.0%
	750	24	2	46	48	95.8%	86.0%	98.8%
	1000	24	2	48	48	100.0%	92.6%	100.0%
NovaSeq 6000 (SP flow cell)	250	22	2	38	44	86.4%	73.3%	93.6%
	500	24	2	48	48	100.0%	92.6%	100.0%
	750	24	2	46	48	95.8%	86.0%	98.8%
	1000	24	2	48	48	100.0%	92.6%	100.0%
NovaSeq 6000 (S4 flow cell)	250	22	2	38	44	86.4%	73.3%	93.6%
	500	24	2	48	48	100.0%	92.6%	100.0%
	750	24	2	46	48	95.8%	86.0%	98.8%
	1000	24	2	48	48	100.0%	92.6%	100.0%

The LOD of the Illumina COVIDSeq Test using the Qiagen extraction method was determined by serial dilution of heat inactivated SARS-CoV-2 virus with known concentration (ATCC VR-1986HK) into pooled negative clinical matrix (nasopharyngeal swab specimen) to 1500 copies per ml, 1000 copies per ml, 750 copies per ml, and 500 copies per ml. The LOD using Qiagen extraction was determined to be 1000 copies per ml.

Sequencing System	ATCC Heat Inactivated Virus (copies per ml)	Replicate Breakdown				Detection Rate		
		Qiagen Extractions	Library Preparations	Detected	Total	Percent Detected	Lower CI	Upper CI
NextSeq 550 (High output)	500	22	2	41	44	93.2%	81.8%	97.7%
	750	24	2	32	48	66.7%	52.5%	78.3%
	1000	24	2	48	48	100.0%	92.6%	100.0%
	1500	24	2	46	48	95.8%	86.0%	98.8%

Sequencing System	ATCC Heat Inactivated Virus (copies per ml)	Replicate Breakdown		Detection Rate				
		Qiagen Extractions	Library Preparations	Detected	Total	Percent Detected	Lower CI	Upper CI
NovaSeq 6000 (SP flow cell)	500	22	2	40	44	90.9%	78.8%	96.4%
	750	24	2	31	48	64.6%	50.4%	76.6%
	1000	24	2	48	48	100.0%	92.6%	100.0%
	1500	24	2	46	48	95.8%	86.0%	98.8%
NovaSeq 6000 (S4 flow cell)	500	22	2	42	44	95.5%	84.9%	98.7%
	750	24	2	32	48	66.7%	52.5%	78.3%
	1000	24	2	48	48	100.0%	92.6%	100.0%
	1500	24	2	46	48	95.8%	86.0%	98.8%

Inclusivity

In silico primer analysis and synthetic short read analysis were performed to evaluate the inclusivity of the Illumina COVIDSeq Test. For primer analysis, SARS-CoV-2 sequences available on GISAID (1382 sequences) and NCBI (162 sequences) were evaluated. A BLASTn (NCBI) analysis was performed to quantify the level of primer homology across these sequences by querying each individual SARS-CoV-2 primer sequence against the downloaded SARS-CoV-2 sequences.

The following table below summarizes the homology analysis for all 1544 SARS-CoV-2 sequences.

Percent of Primer Pairs Homology	Mean	Median	5th percentile	95th percentile
100% homology	97.1%	98.0%	93.9%	100%
>80% homology	97.8%	98.0%	94.9%	100%

In summary, the Illumina COVIDSeq Test has excellent coverage across known strains of SARS-CoV-2.

Cross-Reactivity

In silico analyses were performed to evaluate the cross-reactivity of the Illumina COVIDSeq Test with representative common respiratory pathogens. For the primer analysis, 38 non SARS-CoV-2 consensus genomes were downloaded from NCBI as the negative sample cohort. A BLASTn (NCBI) analysis was then performed to quantify the number of primer pairs with more than 80% homology with each of the genomes in the cohort.

The following table shows the results from the analysis.

Pathogen Name	NCBI Accession Number	Number of Primer Pairs With Homology Above 80%
Adenovirus (e.g. C1 Ad. 71)*	AC 000017 (1) AC 000007 (2) AC_000008 (5) AC 000018 (7) AC 000019 (35) NC_012959(54)	0
Human Metapneumovirus (hMPV)	NC_039199	0
Parainfluenza virus 1	NC_003461	0
Parainfluenza virus 2	NC_003443	0
Parainfluenza virus 3	NC_001796	0

Pathogen Name	NCBI Accession Number	Number of Primer Pairs With Homology Above 80%
Parainfluenza virus 4	NC_021928	0
Influenza virus A	NC_026438	0
Influenza virus B	NC_002204	0
Respiratory syncytial virus	NC_001803	0
Enterovirus (e.g. EV68)	NC_038308	0
Rhinovirus*	NC_038311 (1) NC_038312 (B3) NC_001490 (B14) NC_009996 (C)	0
Human coronavirus 229E	NC_002645	0
Human coronavirus OC43	NC_006213	0
Human coronavirus HKU1	NC_006577	0
Human coronavirus NL63	NC_005831	0
SARS-coronavirus	NC_004718	8
MERS coronavirus	NC_019843	0
Chlamydia pneumoniae	NC_005043	0
Haemophilus influenzae	NZ_LN831035	0
Legionella pneumophila	NZ_LR134380	0
Mycobacterium tuberculosis	NC_000962	0
Streptococcus pneumoniae	NZ_LN831051	0
Streptococcus pyogenes	NZ_CP007593	0
Bordetella pertussis	NC_018518	0
Mycoplasma pneumoniae	NZ_CP010546	0
Pneumocystis jirovecii (PJP)	NJFV01000001 - NJFV01000219	0
Candida albicans	NC_032089 - NC_032096	0
Pseudomonas aeruginosa	NC_002516	0
Staphylococcus epidermis	NZ_CP035288 - NZ_CP035290	0
Streptococcus salivarius	NZ_LR134274	0

* Aggregated results for testing of six different human adenovirus (1, 2, 5, 7, 35, 54) and four different rhinoviruses (1, B3, B14, C).

All pathogens were determined to have no cross-reactivity with the primers used except for SARS-coronavirus (SARS-2003), which has a small portion of primer pairs (8/98) that are potentially cross-reactive. However, these eight potentially cross-reactive primer pairs would not lead to false positive detection of SARS-2003 due to the exclusion of ambiguous k-mer(s) in the detection algorithm.

An analysis of simulated short reads was also performed on the same negative cohort.

The following table shows the results of this analysis and indicates no cross-reactivity of the Illumina COVIDSeq Test[†] with the respiratory pathogens tested.

RefSeq Record Name (Accession Number)	Number of ARTIC Amplicons Detected	COVID-19 Detected
Streptococcus pneumoniae (NZ_LN831051)	0	NO
Human respirovirus 3 (NC_001796)	0	NO
Human adenovirus 1 (AC 000017)	0	NO

RefSeq Record Name (Accession Number)	Number of ARTIC Amplicons Detected	COVID-19 Detected
Haemophilus influenzae (NZ_LN831035)	0	NO
Streptococcus salivarius (NZ_LR134274)	0	NO
Human adenovirus 5 (AC_000008)	0	NO
Human adenovirus 54 (NC_012959)	0	NO
Chlamydia pneumoniae TW-183 (NC_005043)	0	NO
Staphylococcus epidermidis (NZ_CP035288 - NZ_CP035290)	0	NO
Human adenovirus 2 (AC_000007)	0	NO
Rhinovirus C (NC_009996)	0	NO
Influenza B virus (B/Lee/1940) (NC_002204)	0	NO
Human respirovirus 1 (NC_003461)	0	NO
Mycobacterium tuberculosis H37Rv (NC_000962)	0	NO
Human rhinovirus A1 (NC_038311)	0	NO
Rhinovirus B14 (NC_001490)	0	NO
Bordetella pertussis 18323 (NC_018518)	0	NO
Candida albicans SC5314 (NC_032089 - NC_032096)	0	NO
Human coronavirus 229E (NC_002645)	0	NO
Influenza A virus (A/California/07/2009(H1N1)) (NC_026438)	0	NO
Human coronavirus OC43 (NC_006213)	0	NO
Pneumocystis jirovecii (NJFV01000001 - NJFV01000219)	0	NO
Streptococcus pyogenes (NZ_CP007593)	0	NO
Human rhinovirus B3 (NC_038312)	0	NO
Middle East respiratory syndrome-related coronavirus (NC_019843)	0	NO
Human parainfluenza virus 4a (NC_021928)	0	NO
Human metapneumovirus (NC_039199)	0	NO
Enterovirus D68 (NC_038308)	0	NO
Pseudomonas aeruginosa PAO1 (NC_002516)	0	NO
Mycoplasma pneumoniae (NZ_CP010546)	0	NO
Respiratory syncytial virus (NC_001803)	0	NO
Human coronavirus NL63 (NC_005831)	0	NO
Human adenovirus 35 (AC_000019)	0	NO
Human rubulavirus 2 (NC_003443)	0	NO
Severe acute respiratory syndrome-related coronavirus (NC_004718)	0	NO
Human adenovirus 7 (AC_000018)	0	NO
Legionella pneumophila (NZ_LR134380)	0	NO
Human coronavirus HKU1 (NC_006577)	0	NO

Clinical Evaluation

78 clinical nasopharyngeal swab (NP) specimens were tested using the Zymo extraction method, including 40 SARS-CoV-2 positive and 38 SARS-CoV-2 negative specimens. The positive and negative percent agreement are indicated in the following table.

Zymo Extraction Method

EUA RT-PCR Comparator	Illumina COVIDSeq Test Results (NP Swabs)			
	Positive	Negative	Invalid	% agreement (95% CI)
Positive	40	0	0	100%
Negative	1 ¹	36	1 ²	97.3% (85.84-99.3%)

¹ Root cause analysis revealed potential low viral titer specimen identified using the Zymo extraction method.

² One invalid specimen was excluded from analysis.

Clinical performance of the Illumina COVIDSeq Test was evaluated in comparison with an RT-PCR assay authorized by the FDA for use under Emergency User Authorization (EUA RT-PCR). 94 clinical nasopharyngeal swab (NP) specimens were tested using the Qiagen extraction method, including 54 SARS-CoV-2 positive and 40 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.

Qiagen Extraction Method

EUA RT-PCR Comparator	Illumina COVIDSeq Test Results (NP Swabs)			
	Positive	Negative	Invalid	% agreement (95% CI)
Positive	53	1 ¹	0	98.1% (90.2-99.7%)
Negative	1 ¹	38	1 ²	97.4% (86.8-99.5%)

¹ Root cause analysis revealed labeling error.

² One invalid specimen was excluded from analysis.

FDA SARS-CoV-2 Reference Panel Testing

The evaluation of sensitivity and MERS-CoV cross-reactivity was performed using reference material (T1), blinded samples, and a standard protocol provided by the FDA. The study included a range finding study and a confirmatory study for LoD. Blinded sample testing was used to establish specificity and to confirm the LoD. The extraction method and instrument used were QIAamp Viral RNA Mini Kit and NextSeq 550Dx (in RUO mode). The results are summarized in the following table.

Table 9 Summary of LoD Confirmation Result Using the FDA SARS-CoV-2 Reference Panel

Reference Materials Provided by FDA	Specimen Type	Product LoD	Cross-Reactivity
SARS-CoV-2	Nasopharyngeal Swab	5400 NDU/ml	N/A
MERS-CoV		N/A	ND

- ▶ NDU/ml—RNA NAAT detectable units/ml.
- ▶ N/A— Not applicable.
- ▶ ND— Not detected.

References

- 1 Coronavirus. World Health Organization. <https://www.who.int/health-topics/coronavirus/coronavirus>. Accessed May 25, 2020.

- 2 **Testing for COVID-19.** Centers for Disease Control and Prevention. <https://www.cdc.gov/coronavirus/2019-ncov/symptoms-testing/testing.html>. Published May 1, 2020. Accessed May 25, 2020

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