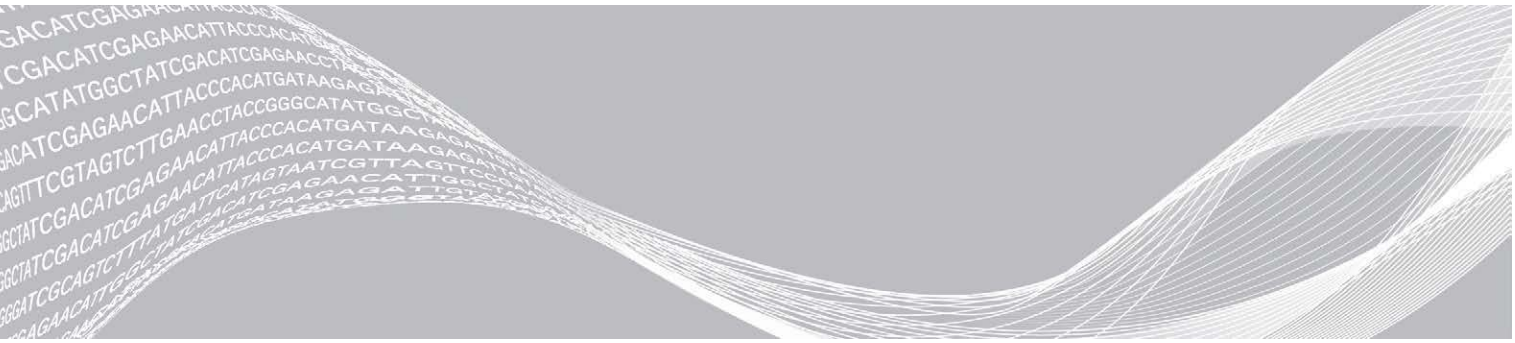
illumina®

Illumina COVIDSeq Test

Reference Guide



Document # 1000000126053 v04

ILLUMINA PROPRIETARY

April 2021

For Research Use Only. Not for use in diagnostic procedures.

Revision History

Document	Date	Description of Change
Document # 1000000126053 v04	April 2021	Added information about variant analysis software options to the Introduction and Prepare for Sequencing section. Added reference to technical note for performing library prep multiple times for a low throughput batch size. Updated the Illumina COVIDSeq Test (3072 samples) from an eight box configuration to a four box configuration in the Kit Contents section. Updated the Read Length recommendations in the Prepare for Sequencing section. Updated Thermal Cycler recommendations for confirmation of microtiter plate compatibility.
Document # 1000000126053 v03	February 2021	Added instructions to set up analysis in BaseSpace Sequence Hub for NextSeq 2000 and other NextSeq 2000-specific information throughout the protocol. Added Thermal Cycler recommendations. Updated materials, consumables, and equipment part numbers in the Prepare for Sequencing, Product Components, and Consumables and Equipment sections, including new consumables for the NextSeq 2000. Updated version numbers of flow cells and control software for the NovaSeq 6000 system. Updated the tube for Dilution 3 in Extract RNA to 5 ml LoBind tube. Updated the protocol options for Quick-DNA/RNA Viral MagBead Procedure for precision and clarity. Updated temperature in PCR program on the thermal cycler for Amplify cDNA Preparation from 65°C to 63°C.
Document # 1000000126053 v02	July 2020	Added instructions for extracting RNA using the Quick-DNA/RNA Viral Magbead kit. Added safe stopping point after pooling and cleaning up libraries. Updated index kit configurations to IDT for Illumina-PCR Indexes. Removed sequencing instructions. Added dilution and sequencing preparation instructions for the NovaSeq 6000 Sequencing System SP flow cell, NextSeq 500 Sequencing System, NextSeq 550 Sequencing System, and NextSeq 550Dx Instrument. Moved data analysis information to *Illumina COVIDSeq Test Pipeline Software Guide document # 1000000128122*.
Document # 1000000126053 v01	June 2020	No content changes.
Document # 1000000126053 v00	June 2020	Initial release.

Table of Contents

Chapter 1 Overview

Introduction

This guide explains how to detect the SARS-CoV-2 virus using the Illumina COVIDSeq Test.

The Illumina COVIDSeq Test offers:

- RNA extraction from decontaminated nasopharyngeal (NP), oropharyngeal (OP), and nasal swab samples, as well as mid-turbinate specimens collected from individuals who meet COVID-19 clinical or epidemological criteria, using the QIAamp Viral RNA Mini Kit or Quick-DNA/RNA Viral Magbead Kit.
- Preparation of up to 3072 samples for high-throughput sequencing using the NovaSeq 6000 Sequencing System or up to 384 samples using the NextSeq 500/550 Sequencing Systems, NextSeq 550Dx Instrument in RUO mode, or NextSeq 2000 Sequencing System.
- Qualitative detection of SARS-CoV-2 RNA using either the Illumina DRAGEN COVIDSeq Test Pipeline locally or with the Illumina DRAGEN COVIDSeq Test app on BaseSpace Sequence Hub.

You can also perform surveillance using either the DRAGEN COVID Pipeline with the COVID Lineage Tools locally or with the DRAGEN COVID Lineage app on BaseSpace Sequence Hub.

Input Recommendations

The Illumina COVIDSeq Test supports patient samples derived from nasopharyngeal (NP), oropharyngeal (OP), and nasal swabs. Transport samples according to the governing regulations for the transport of etiologic agents applicable to your region.

Store samples according to the instructions from the manufacturer. Exceeding the storage times can negatively impact test results.

The following sample factors might affect SARS-CoV-2 detection:

- Sample collection methods, patient factors, and/or the stage of the infection.
- Viral RNA degradation during shipping and storage. RNA degradation can produce false-negative results.

CAUTION

Handle all specimens as infectious reagents.

Chapter 2 Library Prep

Introduction

This chapter describes library preparation using the Illumina COVIDSeq Test.

- Confirm kit contents and make sure that you have the required equipment and consumables. See *Supporting Information* on page 18.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- Make sure reagents are not expired. Using expired reagents might negatively affect performance.
- If performing library prep multiple times for a low throughput (LT) batch size (ie, 96 samples or fewer), refer to the Illumina Technical Note *Aliquot Procedure for Illumina COVIDSeq Test (RUO version) Kit Reagents*.
- 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 -65°C.
- Do not allow more than 8 freeze-thaw cycles for all reagents, excluding CPC HT.
- Include one no template control (NTC) and one positive control per 96-well plate. The internal process control is included in the Illumina COVIDSeq Test.
- Sequence libraries as soon as possible after pooling. Pooled libraries are stable for up to 30 days at -25°C to -15°C.

Tips and Techniques

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

Avoiding Contamination

- Use proper laboratory practices to prevent nuclease and PCR product 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.

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 x 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
- [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 5 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* from Zymo Research.
Use the following protocol options:
- Before adding MagBinding Beads, pipette up and down ten times to mix.
- After adding 20 µl MagBinding Beads, pipette up and down ten times to mix, and then shake at 1500 rpm for 10 minutes.

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).

2 To extract RNA, use the QIAamp Viral RNA Mini Kit. For information, see *QIAmp 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)
- 96-well PCR Plate
- Microseal 'B' adhesive seals

About Reagents

- Vortex before each use

Preparation

1 Prepare the following consumables:

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

2 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

1 Label new PCR plate CDNA1.

2 Add 8.5 µl EPH3 HT to each well.

3 Add 8.5 µl eluted sample to each well.

4 Seal and shake at 1600 rpm for 1 minute.

5 Centrifuge at 1000 × g for 1 minute.

6 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
  - 63°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 CNDA1 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 x 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 × 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 × 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 prepaired 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 x 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 wash 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 the TAG1 plate 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 tube 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.

$$\frac{Library\ concentration\ ng/\mu l}{660\ \frac{g}{mol}\ x\ average\ library\ size\ (bp)} x10^6 = Molarity\ (nM)$$

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 HO flow cell, NextSeq 550Dx HO flow cell, or NextSeq 1000/2000 P2 flow cell: 384 samples per flow cell.
   - NoveSeq 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)*.
   - For the NextSeq 2000 Sequencing System, see the *NextSeq 1000/2000 Sequencing System Guide (document # 1000000109376)*.

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
NextSeq 1000/2000 P2 Flow Cell	4	1000

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

Prepare for Sequencing

The Illumina COVIDSeq Test is compatible with the NovaSeq 6000 Sequencing System SP and S4 flow cells, the NextSeq 2000 Sequencing System, the NextSeq 500/550 Sequencing Systems, and the NextSeq 550DX instrument.

Consumables

- If using the NovaSeq 6000 Sequencing System S4 flow cell:
  - 2 NovaSeq 6000 Sequencing System S4 Reagent Kit v1.5 (35 cycles), Illumina, # 20044417
  - 2 NovaSeq Xp 4-Lane Kit v1.5, Illumina, # 20043131
- If using the NovaSeq 6000 Sequencing System SP flow cell:
  - 4 NovaSeq 6000 Sequencing System SP Reagent Kit v1.5 (100 cycles), Illumina, # 20028401
  - 4 NovaSeq Xp 2-Lane Kit v1.5, Illumina, # 20043130
- If using the NextSeq 500/550 System or NextSeq 550Dx Instrument:
  - 8 NextSeq 500/550 High Output Kit v2.5 (75 Cycles), Illumina, # 20024906
- If using the NextSeq 2000 Sequencing System

- 8 NextSeq 1000/2000 P2 Reagents (100 Cycles), Illumina, # 20046811

Sample Sheet Requirements

The Illumina DRAGEN COVIDSeq Test Pipeline requires a sample sheet for each run analysis. This requirement does not apply to the NextSeq 2000, which uses the Illumina DRAGEN COVIDSeq Test in BaseSpace Sequence Hub.

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: • 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 *Illumina Adapter Sequences (document # 1000000002694)* 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 *Illumina Adapter Sequences (document # 1000000002694)* 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 *Illumina Adapter Sequences (document # 1000000002694)* 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, NextSeq 500Dx, or NextSeq 2000, 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, NextSeq 500Dx, or NextSeq 2000 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`.

5 Save your sample sheet.

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 v1.7 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 the appropriate read length. Refer to the Illumina Technical Note *Sequencing Guidelines for COVID-19 Surveillance Using the Illumina COVIDSeq Test (RUO Version)* for guidance.
  - **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 NexSeq 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 the appropriate read length. Refer to the Illumina Technical Note *Sequencing Guidelines for COVID-19 Surveillance Using the Illumina COVIDSeq Test (RUO Version)* for guidance.
  - **Index 1** and **Index 2**—Enter 10 as the value.

3 If using the NextSeq 2000, refer to the *NextSeq 1000/2000 Sequencing System Guide (document # 1000000109376)*.
- When creating a run in BaseSpace Sequence Hub, make sure to do the following:
  - Select **BaseSpace** for analysis location.
  - Select **Illumina DRAGEN COVIDSeq Test** for analysis type.
  - If Illumina DRAGEN COVIDSeq Test does not appear as an analysis type, contact Illumina Technical Support.
  - Set up the analysis as described in the following *Set Up Analysis in BaseSpace Sequence Hub for NextSeq 2000* section.
- Use v1.2 of the NextSeq 1000/2000 Control Software.
- Make sure **Online Run Setup** and **Proactive, Run Monitoring, and Storage** are selected in the Settings screen to enable Cloud mode.

After sequencing completes, analysis either takes place on your system using installed pipeline software or in BaseSpace Sequence Hub.

- Local analysis for qualitative detection of SARS-CoV-2 RNA uses the Illumina DRAGEN COVIDSeq Test Pipeline.

- Local analysis for surveillance uses the Illumina DRAGEN COVID Pipeline with COVID Lineage Tools.
- Analysis in BaseSpace Sequence Hub can use the Illumina DRAGEN COVIDSeq Test for qualitative detection of SARS-CoV-2 or the DRAGEN COVID Lineage app for surveillance.

Refer to one of the following resources for more information.

- *Illumina DRAGEN COVIDSeq Test Pipeline Software Guide (document # 1000000128122)*
- *Illumina DRAGEN COVIDSeq Test App Guide (document # 1000000129548)*
- *Illumina DRAGEN COVID Pipeline Software Guide (document # 1000000158680)*

Set Up Analysis in BaseSpace Sequence Hub for NextSeq 2000

Use the following steps to configure the Illumina DRAGEN COVIDSeq Test analysis in BaseSpace Sequence Hub when using a NextSeq 2000 instrument.

1. To enable fast mode, set the Fast Mode option to **True**.
   Fast mode turns off alignment, variant calling, and consensus sequence FASTA generation to analyze results.
2. To exclude run logs, QC metric files, and other file types, set the Metrics and Logs Datasets option to **False**.
   Setting this option to false improves analysis speed, but the Logs_Intermediates_Lane_* folder is not generated.
3. Identify the location for your positive and no template controls using either the sample ID or well position.
4. Enter the positive control and no template control for each index set.
   - If you used the index set during library preparation, enter the sample ID or well position for the positive and no template controls.
   - If you did not use the index set, enter NA.
5. Select **Submit Run**.

Appendix A Supporting Information

Kit Contents

The Illumina COVIDSeq Test requires Illumina COVIDSeq Test (3072 Samples) and 8 IDT for Illumina-PCR Indexes.

Component	Kit	Catalog #
Library Preparation	Illumina COVIDSeq Test (3072 Samples)	20043675
Indexes	IDT for Illumina-PCR Indexes Sets 1–4 (384 Indexes)	20043137

Illumina COVIDSeq Test

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

Table 1 Illumina COVIDSeq Test Box 1 – 3072 Samples, Part # 20044405

Quantity	Label Volume (ml	Reagent	Description	Storage
1	233	ITB	Illumina Tune Beads	Room temperature
1	56	ST2 HT	Stop Tagment Buffer 2 HT	Room temperature, post-amp environment

Table 2 Illumina COVIDSeqTest Box 2 – 3072 Samples, Part # 20044406

Quantity	Label Volume (ml)	Reagent	Description	Storage
2	6.1	EBLTS HT	Enrichment BLT HT	2°C to 8°C, post-amp environment
1	114	ELB HT	Elution Buffer HT	2°C to 8°C, pre-amp environment
1	10	RSB HT	Resuspension Buffer HT	2°C to 8°C, post-amp environment
1	845	TWB HT	Tagmentation Wash Buffer HT	2°C to 8°C, post-amp environment

Table 3 Illumina COVIDSeq Test Box 3 – 3072 Samples, Part # 20044407

Quantity	Label Volume (ml)	Reagent	Description	Storage
1	14.4	CPP1 HT	COVIDSeq Primer Pool 1 HT	-25°C to -15°C, pre-amp environment
1	14.4	CPP2 HT	COVIDSeq Primer Pool 2 HT	-25°C to -15°C, pre-amp environment
1	45	EPH3 HT	Elution Prime Fragment 3HC Mix HT	-25°C to -15°C pre-amp environment

Quantity	Label Volume (ml)	Reagent	Description	Storage
1	79	EPM HT	Enhanced PCR Mix HT	-25°C to -15°C, pre-amp environment
1	41	FSM HT	First Strand Mix HT	-25°C to -15°C, pre-amp environment
1	100	IPM HT	Illumina PCR Mix HT	-25°C to -15°C, pre-amp environment
1	4.6	RVT HT	Reverse Transcriptase HT	-25°C to -15°C, pre-amp environment
1	38	TB1 HT	Tagmentation Buffer 1 HT	-25°C to -15°C, post-amp environment

Table 4 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, pre-amp 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).

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

Consumables and Equipment

In addition to the Illumina COVIDSeq Test and IDT for Illumina-PCR Indexes, make sure that you have the required consumables and equipment before starting the protocol.

Consumables

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

Consumable	Supplier
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
Lab tissue, low-lint	VWR, catalog # 21905-026, or equivalent
Lint-free alcohol wipe	General lab supplier
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
RNase/DNase-free Disposable Pipetting Resovoirs	VWR, part # 89094-658
One of the following, depending on the extraction method used: • 13 QIAamp Viral RNA Mini Kit • 8 Quick DNA/RNA Viral MagBead	• Qiagen, catalog # 52906 • Zymo Research, catalog # R2141
Qubit dsDNA HS Assay Kit	One of the following, depending on kit size: • ThermoFisher Scientific, part # Q32851 • ThermoFisher Scientific, part # Q32854
Qubit Assay Tubes	ThermoFisher Scientific, catalog # Q32856
If using the NovaSeq 6000 Sequencing System S4 flow cell: • 2 NovaSeq 6000 Sequencing System S4 Reagent Kit v1.5 (35 cycles) • 2 NovaSeq Xp 4-Lane Kit v1.5	• Illumina, catalog # 20044417 • Illumina, catalog # 20043131
If using the NovaSeq 6000 Sequencing System SP flow cell: • 4 NovaSeq 6000 Sequencing System SP Reagent Kit v1.5 (100 cycles) • 4 NovaSeq Xp 2-Lane Kit v1.5	• Illumina, catalog # 20028401 • Illumina, catalog # 20043130
If using the NextSeq 500/550 System or the NextSeq 550Dx instrument: • 8 NextSeq 500/550 High Output Kit v2.5 (75 Cycles)	• Illumina, catalog # 20024906
If using the NextSeq 2000 System • 8 NextSeq 1000/2000 P2 Reagents (100 cycles)	• Illumina, # 20046811

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

Equipment	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: • Quick-DNA/RNA Viral MagBead equipment • QIAamp Viral RNA Mini Kit equipment	 • See *Quick-DNA/RNA Viral MagBead Instruction Manual*, Zymo Research • See *QIAamp Viral RNA Mini Handbook (document # HB-0354-006)*, Qiagen
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) • MagnaRack Magnetic Separation Rack	 • Thermo Fisher Scientific, catalog #A13346 • Thermo Fisher Scientific, catalog # CS15000
Microcentrifuge	General lab supplier
Microplate Centrifuge	General lab supplier
One of the following sequencing systems: • NextSeq 500 • NextSeq 550 • NextSeq 550Dx • NextSeq 2000 • 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: • C1000 Touch™ Thermal Cycler with 96–Well Fast Reaction Module • C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module • Veriti 96-well Thermal Cycler • GeneAmp PCR System 9700 Fast Thermal Cycler • Thermal cycler that meets the minimum specification requirements. See *Recommended Thermal Cycler Specifications* on page 22	 • Bio-Rad, part # 1851196 • Bio-Rad, part # 1851197 • Thermo Fisher, catalog # 4375786 • Thermo Fisher, catalog # 4339386
Sealing wedge or roller	General lab supplier
Vortexer	General lab supplier

Recommended Thermal Cycler Specifications

The following are the recommended minimum requirements for a thermal cycler used in the Illumina COVIDSeq Test. Make sure to also confirm compatibility of your PCR plate with the specific thermal cycler you use.

Specification	Minimum Requirement
Lid type	Heated
Temperature range	4°C to 99°C
Format	0.2 mL tubes, 96-well plate
Temperature accuracy	±0.25°C (35°C to 99.9°C)
Tempature uniformity	±0.5°C well-to-well within 30 seconds of arrival at target temperature
Peak ramp rate	At least 1.5°C
Sample ramp rate	± 1.25°C