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EasySeq™

# SARS-CoV-2 (novel coronavirus) Whole Genome Sequencing

NGS library prep by Reverse Complement PCR

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*Version: RC-COVID096-4.01  
Revision date: December 16, 2021*



# Protocol

## For Kit V4.0 and higher



## Contents

Product and Company Information	4
Product Use	5
Kit Content.	5
Needed, but not included	6
General precautions	7
1. Thermocycling program	7
2. Reverse Complement PCR	8
3. Pool, Purify and Sequence	10
APPENDIX 1:	14
ILLUMINA® SEQUENCER PROTOCOLS	14
A: Illumina® MiSeq protocol	14
B: Illumina® NextSeq protocol	15
C: Illumina® MiniSeq protocol	16
D: Illumina® iSeq protocol	17
APPENDIX 2	18
Recommended cDNA synthesis with LunaScript RT Supermix 5x	18
Software tools:	18
JSI – Virseak	18
QIAGEN - CLC Bio	18
Ridom – SeqSphere	18
Open Source GITHUB	18
Illumina® Systems Reference Guides	19
Support:	19
Reference:	20
Legal Notice:	20
Version tracking:	19
Published by:	20

## Product and Company Information

Product name: EasySeq™ SARS-CoV-2 Whole Genome NGS Sequencing kit  
Product Info: [www.nimagen.com/covid19](http://www.nimagen.com/covid19)  
Product use: Research Use Only  
Company: NimaGen BV  
Lagelandseweg 56  
6545 CG Nijmegen, The Netherlands  
Telephone: +31 (0)24 820 02 41  
Email: [info@nimagen.com](mailto:info@nimagen.com) / [techsupport@nimagen.com](mailto:techsupport@nimagen.com)

## Product Use

Multiplex Amplicon based NGS Library preparation for sequencing the genome of SARS-CoV-2 virus. To detect mutations, defining strains, monitoring viral populations for epidemiology and outbreak events.

This Reverse-Complement PCR based library prep contains reagents to generate Illumina® compatible sequencing libraries in a simple, sensitive and robust method for fast and cost-effective WGS of the viral genome.

Recommended Read Depth per sample: 150K-200K clusters  
 Recommended Sequencing Length\*: 151-8-8-151  
 \* Only for NovaSeq: 151-10-10-151

## Kit Content.

**Note: One complete kit consist of three part numbers, to be ordered separately:**

1. NimaGen Part# RC-COV096	Box #	Content
SARS CoV2 WGS Panel A (RC-PCR probe mix, black cap)	1 (Frozen)	Tube 24 µL
SARS CoV2 WGS Panel B (RC-PCR probe mix, red cap)	1 (Frozen)	Tube 24 µL
RC-PCR Probe Dilution Buffer Plus (Blue cap)	1 (Frozen)	Tube 190 µL
AmpliClean Bead solution	2 (Cooled)	Tube 1.7 mL
RC-PCR Low-TE Buffer (yellow cap)	2 (Cooled)	Tubes 1 x 2 mL

2. NimaGen Part# MMHS-25	Box #	Content
PCR 2x Hotstart HiFi Mastermix, compatible with RC-PCR (white cap).	1 (Frozen)	Tubes 2 x 1150 µL

Choose one of the index plates of choice

3. NimaGen Part# IDX96-U0xD	Box #	Content
<p>2 x IDX Index Primer Plates, dried down. Choose one of the 6 available Index Plates (U01D, U02D, U03D, U04D, U05D, U06D) with 96 Unique Dual, 10 bp* Indexes for Illumina®.</p> <p>P/N: IDX96-U01D, IDX96-U02D, IDX96-U03D, IDX96-U04D, IDX96-U05D, IDX96-U06D.</p> <p>Semi-skirted, ABI stye PCR Plates, <b>containing dried down</b>, red coloured, Unique Dual Index primer pairs in each well, ready to use. Index Sequences can be downloaded from the product page (<a href="http://www.nimagen.com/covid19">www.nimagen.com/covid19</a>)</p>	1	<p>2 x 96-well plates</p> <p>(Pre-spotted with Red Colored, Dried Down and Stabilized, Unique Dual Index Primers. Both plates from one set are identical)</p>

*\*All Indexes are Unique by reading 8 bp. Only for NovaSeq we recommend to read 10 bp to minimize risk for Index Hopping*

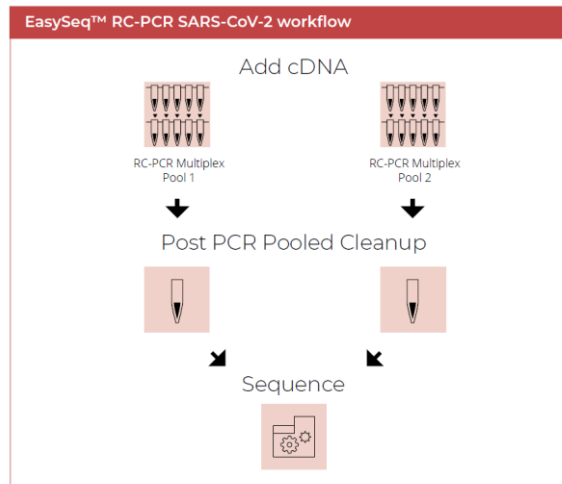
## Needed, but not included

Description	Vendor
Adjustable Pipette Set (P10, P20, P200, P1000)	Multiple Vendors
LunaScript (NEB p/n E3010L) cDNA synthesis kit or LunaScript cDNA synthesis kit (NimaGen p/n LSRT-096). ( <a href="#">download protocol</a> )	New England Biolabs or NimaGen
TapeStation, Bioanalyzer Instrument, or equivalent, incl. High Sensitivity consumables. Or optionally: Agarose Gel (2%)	Agilent® or other
Ethanol absolute, mol. biol. Grade	Multiple Vendors
General plasticware, DNase free (1.5mL / 2.0 mL tubes, pipette tips w/filter)	Multiple Vendors
Ice or tabletop cooling block	Multiple Vendors
Illumina® NGS Sequencing instrument (iSeq, MiSeq, MiniSeq, NextSeq or NovaSeq)	Illumina®
Illumina® Sequencing Reagent kit (min. 2x150 bp run)	Illumina®
Vortex	Multiple Vendors
Mini Spinner for 1.5 / 2.0 mL tubes	Multiple Vendors
Plate Spinner for 96-well plates	Multiple Vendors
Magnetic Stand for 1.5 mL Eppendorf tubes	Multiple Vendors
PCR Grade Water	Multiple Vendors
Qubit™ Fluorometer including dsDNA High Sensitivity kit	ThermoFisher®
2 x Thermocyclers* with heated lid, (0.2 mL standard PCR tubes), compatible with semi-skirted ABI style PCR plates and option for ramp rate programming. Example: Applied Biosystems™ Veriti™, SimpliAmp or MiniAmp Thermal Cycler	Multiple Vendors
The next three items are only necessary when sequencing in-house. (not needed in case of sending samples to a core facility)	
NaOH solution (2N)	Multiple Vendors
Tris/HCl (200 mM), pH 7	Multiple Vendors
Low TE (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA)	Multiple Vendors

\*In case of processing 48 or less samples, 1 Thermocycler is sufficient.

## General precautions

- Use Pre-PCR environment for setting up the RC-PCR reaction. Pooling, cleaning and library preparation should be performed in a Post-PCR environment.
- All reagents need to be thawed and centrifuged before use
- Make sure to mix reagents and reactions properly



## 1. Thermocycling program

Temp:	Duration:	Ramping rate: (from previous step)	Cycles
98°C	2 minutes	N/A	1 x
98°C	10 seconds	Max	1 x
80°C	1 second	Max	
58°C	10 minutes	0.1°C/sec (or 2% of max)	
72°C	2 minute	Max	
95°C	10 seconds	Max	2 x
80°C	1 second	Max	
62°C	90 minutes	0.1°C/sec (or 2% of max)	
72°C	2 minute	Max	
95°C	10 seconds	Max	40 x
80°C	1 second	Max	
62°C	2 minutes	0.5°C/sec (or 10% of max)	
72°C	1 minute	Max	

Heated lid at 105°C

Depending on the instrument, this protocol takes 6-7 hours to complete

**NOTE:** For Applied Biosystems 96-well (0.2 mL) Thermal Cyclers Veriti, SimpliAmp, MiniAmp, download the method files from the product page (section “downloads”), copy to a USB stick and import directly into your cyclers.

## 2. Reverse Complement PCR

*In this step, the target specific RC-probes will be transformed into the functional, tailed and indexed PCR primers, followed by multiplex amplification of the target regions. Starting point is cDNA, obtained from positive extracted viral RNA samples. See [page 18](#) for the protocol to convert extracted RNA to cDNA.*

### 2.1 Thaw on ice:

- SARS CoV2 WGS Panel A probe mix (Black Cap)
- SARS CoV2 WGS Panel B probe mix (Red cap)
- RC-PCR Probe Dilution Buffer Plus (Blue cap)
- PCR 2x Hotstart HiFi Mastermix (White cap)

Centrifuge before use:

- SARS CoV2 WGS Panel A probe mix (Black Cap)
- SARS CoV2 WGS Panel B probe mix (Red cap)

Vortex 3-5 seconds and centrifuge before use:

- RC-PCR Probe Dilution Buffer Plus (Blue cap)
- PCR 2x Hotstart HiFi Mastermix (White cap)

**Note:** *The 2x Hotstart HiFi Mastermix contains isostabilizers and may not freeze completely, even when stored at -15°C to -25°C. It may contain precipitates when thawed at +2°C to +8°C. Always ensure that the Mastermix is fully thawed and thoroughly mixed before use.*

### 2.2 Take the two identical IDX PCR plates and, if needed, cut off the number of strips needed from both plates. Mark the plates with “A” and “B”.

**Note:** *Register the indexes used (IDX set / and well position for each sample). Download the index details for setting up Illumina® samplesheets at the download section of [www.nimagen.com/covid19](http://www.nimagen.com/covid19)*

**Note:** *For each sample, two PCR reactions are needed (pool A and pool B). Always use the same well position for the same sample, in order to generate identical indexes for each sample in both pools.*

### 2.3 Prepare in a new 2 mL tube: Probe-Polymerase premix A, by combining and mixing

- 0.2 µL panel **A** probe mix per reaction (black cap)
- 0.8 µL RC-PCR Probe Dilution Buffer Plus per reaction (blue cap)
- 10 µL PCR 2x Hotstart HiFi Mastermix per reaction (white cap)
- 4 µL H<sub>2</sub>O (Molecular Biology Grade)

### 2.4 Prepare in a new 2 mL tube: Probe-Polymerase premix B, by combining and mixing

- 0.2 µL panel **B** probe mix per reaction (red cap)
- 0.8 µL RC-PCR Probe Dilution Buffer Plus per reaction (blue cap)
- 10 µL PCR 2x Hotstart HiFi Mastermix per reaction (white cap)
- 4 µL H<sub>2</sub>O (Molecular Biology Grade)

**Example: 48 samples + 10% extra volume\***

Tube A:

- 10.56 µL Panel A probe mix (black cap)
- 42.24 µL RC-PCR Probe Dilution Buffer Plus (blue cap)
- 528 µL PCR 2x Hotstart HiFi Mastermix (white cap)
- 211.2 µL H<sub>2</sub>O

Tube B:

- 10.56 µL Panel B probe mix (red cap)
- 42.24 µL RC-PCR Probe Dilution Buffer Plus (blue cap)
- 528 µL PCR 2x Hotstart HiFi Mastermix (white cap)
- 211.2 µL H<sub>2</sub>O

**\*It is recommended to allow for a 10% extra when preparing the mastermix to correct for pipetting loss. The kits contain extra reagent for this.**



- 2.5 Inspect both prespotted IDX plates for colored precipitate in the bottom of each well
- 2.6 Add to each tube of plate A: **15**  $\mu$ L of the Probe-Polymerase premix A
- 2.7 Add to each tube of plate B: **15**  $\mu$ L of the Probe-Polymerase premix B
- 2.8 Add to the same well position of both plates: 5  $\mu$ L (diluted) cDNA

*Example: Add 5  $\mu$ L of cDNA from sample 1 to wells A1 of both plates  $\rightarrow$  total needed: 10  $\mu$ L cDNA*

- 2.9 Close the tube strips or plates carefully with caps (included in the kit) and mix by **vortexing** 3-5 Seconds.
- 2.10 Short spin both plates
- 2.11 Visually check the wells in both plates for even fill volumes. The red dye should be completely and homogeneously dissolved
- 2.12 Start the RC-PCR program in the thermal cyclers and place samples in the cyclers when the blocks are between 60°C and 98°C, close the lid. Program should take 6-7 hours.



*Safe stopping point after RC-PCR. Store at 4°C for max. 48H*

The samples have now been amplified and tagged with sample specific, Unique Dual Indexes and sequencing adapters. From this point, PCR products from both plates will be pooled per plate, as their corresponding pool A and B, purified by an AmpliClean purification to remove primers, primer dimers and salts. In order to decrease read depth variation between samples with low and high viral loads, it is an option to follow a pooling strategy based on the Ct values from the Real-Time PCR assay the samples were detected Covid19 positive with (see table at 3.3).

### 3. Pool, Purify and Sequence

*Note: Before pooling, optionally check 3 µL of the unpurified PCR products on agarose (2%).*

- 3.1 Bring the AmpliClean™ beads solution to Room Temperature.
- 3.2 Mark 2 x 1.5 mL Eppendorf tubes with resp. “A” and “B”.
- 3.3 Create a PCR pool from both plates in resp. tube A and B, by combining 5 µL RC-PCR products from each well of each of the two plates (except negative controls). If a Ct dependent read depth correction is desirable, follow the pooling strategy according to the table below.

Ct value from qPCR	RC-PCR volume
< 20 (very high viral load)	2 µL
20-24 (high viral load)	4 µL
25-28 (medium viral load)	8 µL
> 28 (low viral load)	16 µL

*Note: Keep pool A and B separated during the complete cleanup procedure in two tubes.*

- 3.4 Mix well and transfer 40 µL of both pools to two new 1.5 mL Eppendorf tubes (marked “A2” / “B2”).
- 3.5 Add 60 µL RC-PCR Low TE buffer (yellow cap) to both tubes A2 and B2 (total volumes are now 100 µL each).

## 3.6 Double AmpliClean™ purification:

### Purification #1

- a. Vortex beads thoroughly to resuspend.
- b. Add 85  $\mu\text{L}$  beads solution to the two 100  $\mu\text{L}$  pools A2 and B2 (from step 3.5) and mix well immediately by pipetting up and down 5 times.
- c. Incubate for 5 minutes, off magnet.
- d. Place both tubes on magnet for 3 minutes or for the solutions to be fully cleared.
- e. Remove and discard liquid carefully without disturbing the beads.
- f. Add 300  $\mu\text{L}$  (freshly prepared) 75% ethanol, without disturbing the beads.
- g. Wait for 1 minute.
- h. Repeat steps **e.**, **f.** and **g.** for a second ethanol wash step
- i. Carefully remove all liquid without leaving traces of ethanol. (Optionally a quick spin can be performed, then place tube back on magnet and remove last traces of ethanol)
- j.** Dry with open cap for 2-3 minutes at Room Temperature. **Do not over-dry.**
- k. On Magnet: Add 120  $\mu\text{L}$  RC-PCR Low TE buffer (Yellow cap).
- l. Off Magnet: Re-suspend the beads by pipetting up and down or flicking.
- m. Incubate for 2 minutes, off magnet.
- n. Put on magnet and wait for 3-5 minutes or for the solution to be fully cleared.
- o. Carefully bring 100  $\mu\text{L}$  of the clear solution to two new 1.5 ml Eppendorf tubes (marked "A3" and "B3") making sure not to transfer any of the beads.

### Purification #2

- p. Add 85  $\mu\text{L}$  resuspended beads solution to the two 100  $\mu\text{L}$  pools A3 and B3 and mix well immediately by pipetting up and down 5 times
- q. Incubate for 5 minutes, off magnet.
- r. Place both tubes on magnet for 3 minutes or for the solutions to be fully cleared.
- s. Remove and discard liquid carefully without disturbing the beads.
- t. Add 300  $\mu\text{L}$  (freshly prepared) 75% ethanol, without disturbing the beads.
- u. Wait for 1 minute.
- v. Repeat steps **s.**, **t.** and **u.** for a second ethanol wash step
- w. Carefully remove all liquid without leaving traces of ethanol. (Optionally a quick spin can be performed, then place tube back on magnet and remove last traces of ethanol)
- x. Dry with open cap for 2-3 minutes at Room Temperature. **Do not over-dry.**

### 3.7 Elution

- On Magnet: Add 50  $\mu\text{L}$  RC-PCR Low TE buffer to the two tubes A3 and B3
- Off Magnet: Re-suspend the beads by flicking or short vortexing.
- Incubate for 2 minutes, off magnet.
- Put back on magnet and wait for 3-5 minutes or for the solutions to be fully cleared.
- Carefully bring 40  $\mu\text{L}$  of the clear solution to two new 1.5 ml Eppendorf tubes (marked A4 and B4) making sure not to transfer any of the beads.



*Safe stopping point after 2nd clean-up. Store at  $-20^{\circ}\text{C}$  if not continuing same day.*

Libraries are now ready for quantification and qualification.

3.8 Determine final concentration of both A4 and B4 by a duplo Qubit (HS) measurement according to manufacturer's manual. Recommended dilution for Qubit is 2  $\mu\text{L}$  of purified product plus 198  $\mu\text{L}$  Qubit HS dilution buffer with assay reagent. Use the dedicated SARS-CoV-2 WGS calculator at the download section of [www.nimagen.com/covid19](http://www.nimagen.com/covid19)

#### SARS-CoV-2 Calculator

**Step 3.8:** Download the SARS-CoV-2 WGS calculator and just fill in (at the green fields, step 1.) the concentration of both A4 and B4, in  $\text{ng}/\mu\text{L}$ . The sheet automatically calculates the pipetting schemes for creating 2 nM libraries (step 2), followed by the pipetting scheme for the denaturation (step 3) and creating the final library for the different Illumina instruments / kits (step 4).

### SARS-CoV-2 WGS Calculator

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#### 1. Qubit Concentration calculation

Calculated Qubit Readout:			
pool A:	15.00	$\text{ng}/\mu\text{L}$	= 51.1 nM
pool B:	12.00	$\text{ng}/\mu\text{L}$	= 40.9 nM

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#### 2. Dilution of both pools to 50 $\mu\text{L}$ of 2 nM

	pool volume ( $\mu\text{L}$ )	low TE volume
pool A:	2.0	48.0
pool B:	2.4	47.6

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#### 3. Denaturation pipetting scheme

Pool A (2 nM)	5 $\mu\text{L}$
Pool B (2 nM)	5 $\mu\text{L}$
NaOH (0.2 N)	10 $\mu\text{L}$
<i>incubate 5 minutes at room temp</i>	
Tris-HCl (200 mM)	10 $\mu\text{L}$
Ice Cold Buffer HT1	970 $\mu\text{L}$
<b>Total (20 pM)</b>	<b>1000 <math>\mu\text{L}</math></b>

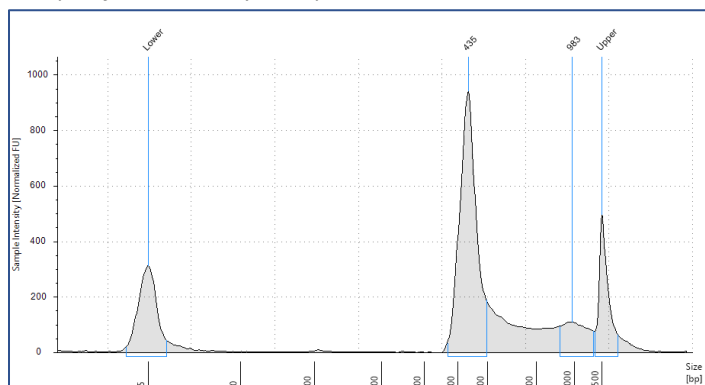
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#### 4. Final Library pipetting scheme

	Loading concentration (pM)	end volume ( $\mu\text{L}$ )	Denaturated pool (20 pM) volume ( $\mu\text{L}$ )	HT1 volume ( $\mu\text{L}$ )	20 pM PhiX control ( $\mu\text{L}$ )
<i>MiniSeq</i>	0.8	500	20	479	1
<i>MiSeq v2</i>	9	600	270	325	5
<i>MiSeq v3</i>	15	600	450	140	10
<i>NextSeq</i>	0.9	1500	67.5	1429.5	3

3.9 Verify library on TapeStation or Bioanalyzer, according to the manufacturer’s protocol. If needed, dilute pool. Example: For TapeStation High Sensitivity kit, dilute to ~2 ng/μL

Example of a clean library on TapeStation:



3.10 Perform Sequencing on an Illumina® NGS platform, according to the manufacturer’s manual.

**NOTE:** Download Index Sequences from [www.nimagen.com/covid19](http://www.nimagen.com/covid19), section “downloads” to create a sample sheet. For technical assistance contact our technical support at [techsupport@nimagen.com](mailto:techsupport@nimagen.com).

## APPENDIX 1:

### ILLUMINA® SEQUENCER PROTOCOLS

*Note: When using an iSeq or NovaSeq instrument, please contact our support at [info@nimagen.com](mailto:info@nimagen.com)*

#### A: Illumina® MiSeq protocol

Use Illumina® MiSeq v2 or v3 kit for 300 cycles (2x150 bp run).

Ref: MiSeq System Denature and Dilute Libraries Guide #15039740 v03.

- 1 Thaw DNA sample/library, buffer HT1, and MiSeq cartridge.

*NOTE: MiSeq cartridge should be thawed by submerging it in (but not covering it completely with) water at room temperature. (thawing takes ~ 1.5hr, do not use warm water as it degrades the enzymes). Store other component of MiSeq kit in 4°C refrigerator until ready to start the MiSeq run.*

- 2 Prepare sample sheet. Workflow: Generate FASTQ. Use the following adapter sequences for trimming in the sample sheet:

Adapter: GCGAATTTTCGACGATCGTTGCATTAAGTCGCGAA

AdapterRead2: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

- 3 Dilute DNA to 2 nM using LowTE (yellow cap)
- 4 Prepare fresh 0.2 N NaOH.
- 5 mix 10 µL of 2 nM DNA with 10 µL of 0.2N NaOH, vortex, spin down.
- 6 Incubate for 5 minutes at Room Temperature.
- 7 Add 10 µL of 200 mM Tris-HCl pH 7.0 to hydrolyse the NaOH.
- 8 Add 970 µL Ice Cold HT1 to DNA/NaOH mix to dilute DNA to 20 pM.
- 9 Dilute the 20 pM library with Ice Cold HT1 to 9 pM (for v2 kits) or 15 pM (for v3 kits) in a new tube:
  - a. Dilute 270 of library (20 pM) with 330 µL of HT1  
(Total volume is now 1000 µL of 9 pM loading concentration)
  - b. Invert to mix and then pulse centrifuge.
- 10 Mix the MiSeq cartridge by inverting 10x, make sure the reagents do not contain ice. After mixing, tap the cartridge on the bench 2-3 times to dislodge any air trapped in the bottom of the tubes.
- 11 Load MiSeq cartridge:
  - a. Optional but recommended, use PhiX control: Add 3% of PhiX control to the library
  - b. Load 600 µL of **the library** to the **Load Samples** well.
- 12 Set up and start MiSeq run: Clean flow cell according to instructions, follow on-screen instructions to load and start instrument.

## B: Illumina® NextSeq protocol

Use Illumina® NextSeq v2 kit for 300 cycles (2x150 bp run)

1. Thaw DNA sample/library, buffer HT1, and NextSeq cartridge

*NOTE: Nextseq cartridge should be thawed by submerging it in (but not covering it completely with) water at room temperature. (thawing takes ~ 1.5hr, do not use warm water as it degrades the enzymes)*

2. Prepare sample sheet. Workflow: Generate FASTQ. Use the following adapter sequences for trimming in the sample sheet:

Adapter: GCGAATTTTCGACGATCGTTGCATTAAGTCGCGAA

AdapterRead2: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

3. Dilute DNA to 2 nM using LowTE (yellow cap)
4. Prepare fresh 0.2 N NaOH
5. Mix 10 µL of 2 nM DNA with 10 µL of 0.2N NaOH, vortex, spin down
6. Incubate for 5 minutes at Room Temperature
7. Add 10 µL of 200mM Tris-HCl pH7.0 to hydrolyze the NaOH
8. Add 970 µL Ice Cold HT1 to DNA/NaOH mix to dilute DNA to 20 pM
9. Dilute the 20 pM library with Ice Cold HT1 to 0.9 pM in a new tube
  - a. Dilute 67.5 µL of library (20pM) with 1432.5 µL of HT1
    - i. (This is now 1500µL of 0.9pM loading concentration)
    - ii. Invert to mix and then pulse centrifuge.
10. Mix the NextSeq cartridge by inverting 10x, make sure the reagents do not contain ice. After mixing, tap the cartridge on the bench 2-3 times to dislodge any air trapped in the bottom of the tubes.
11. Load NextSeq cartridge:
  - a. Optional but recommended, use PhiX control: Add 3% of PhiX control to the library
  - b. Load 1300 µL of **the library** to the **Load Library Here (10)** well
12. Set up and start Nextseq run: Clean flow cell according to instructions, follow on-screen instructions to load and start instrument

## C: Illumina® MiniSeq protocol

Use Illumina® MiniSeq kit for 300 cycles (2x150 bp run)

1. Thaw DNA sample/library, buffer HT1, and MiniSeq cartridge

**NOTE: MiniSeq cartridge should be thawed by submerging it in (but not covering it completely with) water at room temperature. (thawing takes 2-3 hour, do not use warm water as it degrades the enzymes)**

2. Prepare sample sheet. Workflow: Generate FASTQ. Use the following adapter sequences for trimming in the sample sheet:

Adapter: GCGAATTTTCGACGATCGTTGCATTAAGTCGCGAA

AdapterRead2: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

3. Dilute DNA to 2 nM using lowTE (yellow cap).
4. Prepare fresh 0.1N NaOH.
5. Mix 10 µL of 2 nM DNA with 10 µL of 0.1N NaOH, vortex, spin down.
6. Incubate for 5 minutes at room temperature.
7. Add 10 µL of 200 mM Tris-HCl pH 7.0 to hydrolyze the NaOH.
8. Add 970 µL Ice Cold HT1 to DNA/NaOH mix to dilute DNA to 20 pM.
9. Dilute the 20 pM library with Ice Cold HT1 to 0.8 pM in a new tube.
  - a. Dilute 40 µL of library (20 pM) with 960 µL of HT1
  - b. (This is now 1000 µL of 0.8 pM loading concentration)
  - c. Invert to mix and then pulse centrifuge.
10. Mix the MiniSeq cartridge by inverting 10x, make sure the reagents do not contain ice. After mixing, tap the cartridge on the bench 2-3 times to dislodge any air trapped in the bottom of the tubes.
11. Load MiniSeq cartridge:
  - a. Optional but recommended, use PhiX control: Add 3% of PhiX control to the library
  - b. Load 500 µL of **the library** to the **Load Library Here (16)** well
12. Set up and start MiniSeq run: Clean flow cell according to instructions, follow on-screen instructions to load and start instrument



## D: Illumina® iSeq protocol

Use Illumina® iSeq100 kit for 300 cycles (2x150 bp run)

1. Thaw DNA sample/library and iSeq cartridge

**NOTE: MiniSeq cartridge should be thawed by submerging it in (but not covering it completely with) water at room temperature. (thawing takes 2-3 hour, do not use warm water as it degrades the enzymes)**

2. Prepare sample sheet. Workflow: Generate FASTQ. Use the following adapter sequences for trimming in the sample sheet:

Adapter: GCGAATTCGACGATCGTTGCATTAAGCGCGAA

AdapterRead2: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

3. Dilute Pool A and B to 2 nM using lowTE (yellow cap) as per Calculation sheet.
4. Combine 10ul of pool A and B to a final library of 2nM
5. Dilute Final Library to 50 pM.
  - a. 5ul Library 2nM
  - b. 195ul LowTE
6. Dilute PhiX to 1nM
  - a. 10ul PhiX 10nM
  - b. 90 ul LowTE
  - c. Aliquot in 15ul and store in -20 for max 4 weeks.
7. Dilute PhiX to 100pM
  - a. 10ul PhiX 1nM
  - b. 90ul LowTE
8. Prepare Libarry for loading
  - a. 98ul Final library 50pM
  - b. 2ul PhiX 100pM
9. Mix the iSeq cartridge by inverting 10x, make sure the reagents do not contain ice. After mixing, tap the cartridge on the bench 2-3 times to dislodge any air trapped in the bottom of the tubes.
10. Load iSeq cartridge:
  - a. Load 20 µL of **the Library for loading** to the **Library** well
  - b. Load the flowcell in the cardridge
11. Set up and start iSeq run
12. Amplicon library – custom barcodes

## APPENDIX 2

### Recommended cDNA synthesis with LunaScript RT Supermix (5x)

cDNA synthesis using LunaScript™ Reverse Transcription kit of New England Biolabs is the recommended method for reverse transcription of the RNA, isolated from viral samples, because of the compatibility of the buffering system with the buffering system of the HiFi polymerase used in the RC-PCR reaction. Catalog Number E3010S or E3010L (New England Biolabs) or LSRT-096 (NimaGen).

Quick Start Protocol for generation of 11 µL cDNA:

- <i>Lunascript Supermix</i>	2.2 µL
- <i>Water</i>	3.3 µL
- <i>RNA sample</i>	5.5 µL
<b>Total</b>	<b>11 µL</b>

Thermal Profile:

- 2 min	25°C (primer annealing)
- 20 – 45 min	55°C (reverse transcription)
- 1 min	95°C (heat inactivation)
- ∞ 4°C	

Use 2 x 5 µL of this cDNA directly in the RC-PCR reaction according to this protocol, starting at point 2.1

Software tools:

#### JSI – Virseak

<https://www.jsi-medisys.de/company/news-detail/article/find-known-and-new-variants-of-sars-cov-2-with-our-new-surveillance-system-virseak/>

#### QIAGEN - CLC Bio

<https://digitalinsights.qiagen.com/resources/science/sars-cov-2-resources/>

#### Ridom – SeqSphere

<https://www.ridom.de/seqsphere/>

#### Open Source GITHUB

Co-development Nimagen & Radboud University Medical Center

Department of Medical Microbiology - Dr Jordy Coolen

[https://github.com/JordyCoolen/easyseq\\_covid19](https://github.com/JordyCoolen/easyseq_covid19)

## ILLUMINA® SYSTEMS REFERENCE GUIDES

- iSeq100 System Guide
- MiSeq System Guide
- MiSeq Denature and Dilute Libraries Guide
- MiniSeq System Guide
- MiniSeq Denature and Dilute Libraries Guide
- NextSeq 550 System Guide
- NextSeq System Denature and Dilute Libraries Guide
- ILLUMINA® EXPERIMENT MANAGER

## Version tracking:

Version	change	Date
V1.5 -> v1.6	MiSeq v2 loading changed from 5.5 to 9pM MiSeq v3 loading changed from 10 to 15pM EBT buffer → LowTE	19jan2021, WvdV
V1.6 -> v1.7	iSeq100 kit Safe stopping points added	20jan2021, wvdv
V1.7 -> v1.8	Reverse Transcription kit recommendation, safe stopping points info, tube labelling	19feb2021, jth
V1.8 -> v1.9	safe stopping points info, tube labelling	02mar2021, jth
V1.9 -> v1.10	Naming of Probe Dilution Buffer Plus Thaw, mix and centrifuge of reagents before use	02Apr2021, WvdV
V1.10 -> v2.01	Vortexing of complete 20µl RCPCR mixture prior to thermo-profiling. Updated picture of library calculator Addition of index plate UDI04D Addition of Lunascript protocol	09Apr2021, WvdV
V2.01 -> v3.01	Probe pool update	27Oct2021, jth
V3.01 -> v4	For use with SARS-CoV-2 WGS kits of version 4.0 or higher Probe Pool update to optimize for Omicron variant (bed files not changed) IDX plates dried down Amendment to thermal protocol	16DEC2021, jth

## Support:

<https://www.nimagen.com/covid19>

[techsupport@nimagen.com](mailto:techsupport@nimagen.com)

+31 (0)24 8200241

## Reference:

Jordy P.M. Coolen et.al., J.Clin.Vir., (2021). SARS-CoV-2 whole-genome sequencing using reverse complement PCR: For easy, fast and accurate outbreak and variant analysis  
<https://doi.org/10.1016/j.jcv.2021.104993>

## Legal Notice:

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Lagelandseweg 56  
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[www.nimagen.com](http://www.nimagen.com)  
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