Instructions For Use

Version: 6.0 *Ref: IFU_RC-COV* *Revision date: 2026-01-23*

EasySeq™

SARS-CoV-2 (novel coronavirus)

Whole Genome Sequencing

For NGS Library Prep by Reverse Complement PCR

NimaGen.

Innovators in DNA Sequencing Technologies

Product and Company Information

EasySeq™ SARS-CoV-2 (novel coronavirus) Whole Genome Sequencing

REF RC-COV096

Research Use Only

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Symbols Used on Product Labels and in Instruction For Use

Symbol	Description
	Manufacturer
	Use-by date
LOT	Lot number
REF	Reference number
	Temperature limit for storage
	Contains sufficient for <*n*> tests
	GS1 DataMatrix, containing information about the product

Product Description

The intended purpose of this assay is NGS library preparation for illumina® sequencing of the genome of SARS-CoV-2 virus. To detect mutations, defining strains, monitoring viral populations for epidemiology and outbreak events.

The assay provides reagents for Multiplex Amplicon-based NGS library preparation and is for research use only.

The kit is based on the unique and patented Reverse Complement PCR technology, providing a safe, robust and simple workflow, combining amplification with indexing and adapter addition in a single reaction, decreasing the risk of PCR contamination and sample swapping, for fast and cost-effective WGS of the viral genome.

Reverse Complement PCR Kit Contents

NimaGen Part# RC-COV096 (store at -20 °C)	Contents
SARS CoV2 WGS Probe Panel A (REF: PM-SARSCoV2_A)	1x Tube (24 µL)
SARS CoV2 WGS Probe Panel B (REF: PM-SARSCoV2_B)	1x Tube (24 µL)
2x PCR Master Mix (HiFi) (REF: MM096)	2x Tube (1150 µL)
Probe Dilution Buffer enhanced (REF: PDB-Enh-2x)	1x Tube (432 µL)

Required Materials, Not Included

Description	Vendor
Index Primer Plate, dehydrated. Choose one of the 4 available EasySeq™ Unique Dual Index plates for Illumina. Available REF: IDX96-U01D, IDX96-U02D, IDX96-U03D, IDX96-U04D. **Note:** The index sequences are available from the download section of the NimaGen website. **Note:** If more than 384 indexes are desired, please contact NimaGen for the possibilities.	NimaGen
LunaScript (NEB p/n E3010L) cDNA synthesis kit	LunaScript (NEB p/n E3010L) cDNA synthesis kit
Adjustable Pipette Set (P10, P20, P100, P200, P1000)	Multiple Vendors
TapeStation, Bioanalyzer Instrument, incl. consumables. Or optionally: Agarose Gel (2%)	Agilent
Ethanol Absolute, Molecular Biology Grade	Multiple Vendors
AmpliClean™ or AMPure XP Bead Solution	NimaGen / Beckman Coulter
General plasticware, DNAse free (1.5 mL tubes, pipette tips etc.)	Multiple Vendors
Mini Spinner for 1.5 mL tubes and 8-well PCR strips or PCR plates	Multiple Vendors
Magnetic stand for 1.5 mL tubes and/or 96-wells plates	Multiple Vendors / NimaGen
Molecular grade H_2O	Multiple Vendors
Qubit Fluorometer incl. dsDNA High Sensitivity consumables	Thermo Fisher Scientific
Thermal cycler with heated lid, (0.2 mL standard PCR tubes), compatible with semi-skirted ABI style PCR plates and option for ramp rate programming. **Note:** Kit is validated for Applied Biosystems™ Veriti™, MiniAmp™ and SimpliAmp™ Thermal Cyclers.	Multiple Vendors
NaOH Solution (2 N), NGS grade	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
Illumina NGS Sequencing Instrument (MiSeq®/NextSeq®/MiniSeq®)	illumina®
Illumina MiSeq® Reagent kit.	illumina®

General Precautions

Read the Material Safety Data Sheet (MSDS) and follow the handling instructions. Adhere to good laboratory practice when handling both the reagents supplied in this kit and other reagents required.

Use a Pre-PCR environment for setting up the RC-PCR. Sample pooling, purification and library quantification 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.

Make sure to place the remaining strip(s) of the index plate(s) back into the provided plastic bag and include the silica gel pouch. Try to leave as little air as possible inside the plastic bag, while sealing it.

Protocol

1. Thermal Cycling Program

Temp	Duration	Ramp 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 minutes	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	
16 °C	∞	Max	1 x

Heated lid at 105 °C.

***Note:** Use a max ramp rate of 4 °C/sec. If this rate is not an option for your thermal cycler, choose the highest ramp rate possible.
Note: This protocol takes approximately 6-7 hours to complete, but may vary per thermal cycler used. When running this protocol for the first time, start the cycling program as a dummy run, to check the predicted duration of 6-7 hours.

2. Reverse Complement PCR

In a single, closed tube reaction, the target specific RC-probes are working as a template to extend the UDI primers to synthesize functional, tailed and indexed PCR primers. This will be followed by two long hybridization/extension steps of 90 minutes and subsequently a further DNA amplification of the target regions, meanwhile synthesizing more primers.

2.1 Thaw on ice:
- RC-PCR Probe Panel A (Black cap)
- RC-PCR Probe Panel B (Red cap)
- Probe Dilution Buffer (Blue cap)
- HiFi Master Mix (Purple cap)

Note: The HiFi Master Mix contains iso-stabilizers 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 Master Mix is fully thawed and thoroughly mixed before use.

2.2. Take two identical IDX PCR plates and break off the number of strips needed. Mark the plates with 'A' and 'B'.

Note: Register the indexes used (IDX set/strip-column number and well position for each sample). Download the index details for setting up the Illumina sample sheet.

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

Note: Before breaking off 8-well strips, cut the seal at the breaking line with a sharp knife.

2.3. Prepare in a fresh 1.5 mL tube the RC-PCR mix panel A by combining and mixing:
- 0.2 µL RC-PCR Probe Panel A per reaction (Black cap)
- 0.8 µL Probe Dilution Buffer per reaction (Blue cap)
- 4 µL Water (Molecular Biology Grade)
- 10 µL HiFi Master Mix per reaction (Purple cap)

2.4. Prepare in a fresh 1.5 mL tube the RC-PCR mix panel B by combining and mixing:
- 0.2 µL RC-PCR Probe Panel B per reaction (Red cap)
- 0.8 µL Probe Dilution Buffer per reaction (Blue cap)
- 4 µL Water (Molecular Biology Grade)
- 10 µL HiFi Master Mix per reaction (Purple cap)

Example: 24 samples + 10% extra volume*
- 5.28 µL RC-PCR Probe Panel
- 21.12 µL Probe Dilution Buffer
- 105.6 µL Water
- 264 µL HiFi Master Mix

*It is recommended to allow for a 10% excess when preparing the RC-PCR mix to correct for any pipetting loss. The kit contains extra reagent to facilitate this.

2.5. Remove the seal from the PCR plate or strip(s).

2.6. Dispense 15 µL of the RC-PCR mix Panel A (from step 2.3) to each well of the plate/strip(s) A.

2.7. Dispense 15 µL of the RC-PCR mix Panel B (from step 2.4) to each well of the plate/strip(s) B

2.8. Add to each well either 5 µL of (diluted) cDNA solution.

Example: Add 5 µL of cDNA from sample 1 to wells A1 of both plates → total needed: 10 µL cDNA

2.9. Close the tube strips **firmly** with the caps provided. Mix by short vortexing, followed by a quick spin. Verify that the colour of the reaction mix is homogeneously pink.

2.10. Place the samples in the thermal cycler(s) and start the RC-PCR program.

After the RC-PCR, samples have been amplified and tagged with sample-specific indexes and sequencing adapters. From this point, RC-PCR product purification is performed using a magnetic bead based purification to remove primers, dimers and salts.

Safe stopping point: After completion of the RC-PCR you can store the reactions for up to 48 hours at 4 °C. For longer storage, keep the samples at -20 °C. It is best to continue with the samples within a month.

3. Purification

The purification involves one-sided size selection using magnetic beads, minimizing the number of sequencing reads lost to residual primers and primer-dimers. The quality and quantity of your input samples will impact the PCR yield. Samples can be pooled based on the total input quantity of the PCR to ensure low-input samples have appropriate read depth.

3.1. Bring the magnetic bead solution (AmpliClean™ or AMpure XP) to **room temperature.**

Note: It is important to first let the magnetic bead solution equilibrate to room temperature before continuing with the purification. A colder bead solution will lead to inaccurate size selection.

3.2. Perform steps 3.3 to 3.7 for both Panel A and Panel B individually.

3.3. Pool 5 µL RC-PCR product from each reaction into a 1.5 mL tube. 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: Perform all subsequent steps for each pool individually.

Note: Before pooling, optionally check the unpurified PCR products on agarose.

3.4. Mix well and transfer 40 µL of this pool into a new 1.5 mL tube.

3.5. Add 60 µL Low TE buffer or molecular grade H_2O to the tube and mix well (total volume is now 100 µL).

3.6. Beads purifications:

Purification #1

a. Vortex the beads (AmpliClean™ or AMPure XP) thoroughly to resuspend.
b. Add 85 µL bead solution to the 100 µL pool (from step 3.5) and mix well immediately by pipetting up and down at least 5 times or by short vortexing.
c. Incubate for 5 minutes.

On magnet:
d. Place the tube for 3 minutes on the magnet, or until the solution is fully cleared.
e. Remove and discard the liquid carefully, without disturbing the beads.
f. Add 300 µL (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. (Optional: quick spin, then place the tube **back on the magnet** and remove the last traces of ethanol).
j. Dry with open cap for 2-3 minutes at room temperature. **Do not over-dry.**
k. Add 110 µL Low TE buffer.

Off magnet:
l. Resuspend the beads by pipetting up and down, by flicking or by short vortexing.
m. Incubate for 2 minutes.

On magnet:
n. Wait for 3-5 minutes, or until the solution is fully cleared.
o. Carefully bring 100 µL of the clear solution into a new 1.5 mL tube, ensuring not to transfer any of the beads.

Purification #2

Off magnet:
p. Vortex the beads (AmpliClean™ or AMpure XP) thoroughly to resuspend.
q. Add 85 µL bead solution to the 100 µL pool (from step **o.**) and mix well immediately by pipetting up and down at least 5 times or by short vortexing.
r. Incubate for 5 minutes.

On magnet:
s. Place the tube for 3 minutes on the magnet, or until the solution is fully cleared.

t. Remove and discard the liquid carefully, without disturbing the beads.
u. Add 300 µL (freshly prepared) 75% ethanol, without disturbing the beads.
v. Wait for 1 minute.

w. Repeat steps **s.**, **t.** and **u.** for a second ethanol wash step.
x. Carefully remove all liquid without leaving traces of ethanol. (Optional: quick spin, then place the tube **back on the magnet** and remove the last traces of ethanol).
y. Dry with open cap for 2-3 minutes at room temperature. **Do not over-dry**. Immediately continue with the Elution.

3.7. Elution:

a. On magnet: Add 50 µL Low TE buffer to the tube and close the tube.
b. Off magnet: Resuspend the beads by flicking, or by short vortexing.
c. Off magnet: Incubate for 2 minutes.
d. On magnet: Wait for 3-5 minutes, or until the solution is fully cleared.
e. On magnet: Carefully bring 40 µL of the clear solution into a new 1.5 mL tube, making sure not to transfer any of the beads.

The libraries are now ready for a quantitative and qualitative check, followed by NGS.

Safe stopping point: After the 2nd purification you can store the libraries at 4 °C for up to a week. For longer storage, keep the samples at -20 °C. It is best to sequence the library within a month. Perform a fresh quantification (see 4.1) after storing the library for longer than a day at either temperature.

4. Sequencing

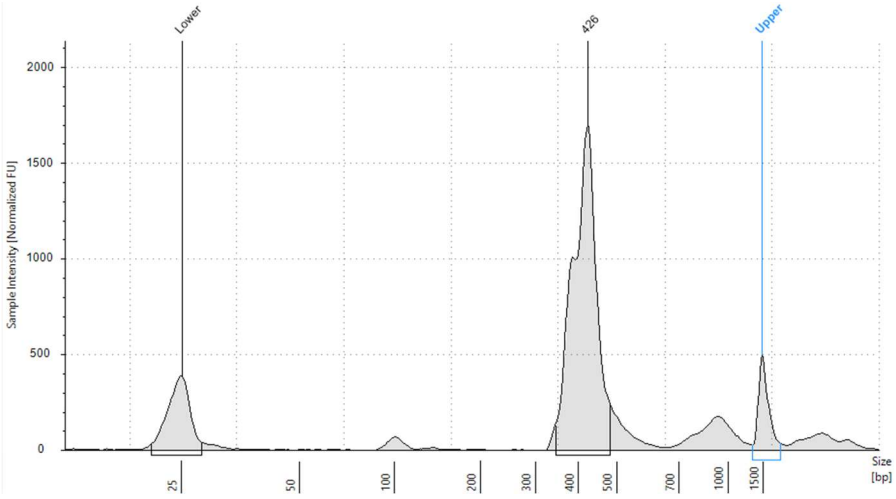
4.1. Determine the final concentration of the libraries by a double Qubit (HS) measurement:

- **a.** Bring the Qubit reagents to room temperature.
- **b.** Label the Qubit tubes on the lid according to the number of samples to be used plus 2 standards.
- **c.** Dilute the Qubit dsDNA HS Reagent 1:200 in Qubit dsDNA HS Buffer for each sample/ standard. It is recommended to allow for >10% excess when preparing the working solution to correct for any pipetting loss.
- **d.** For the standards: mix 190 µL of the working solution with 10 µL of the standard.
- **e.** For the samples: mix 180-199 µL of the working solution with 1-20 µL sample (total 200 µL).
- **f.** Vortex the tubes thoroughly and incubate the tubes for 2 minutes.
- **g.** Measure the standards and the samples using the 'dsDNA High Sensitivity' settings making sure to select the correct sample volume used in step **e.**.

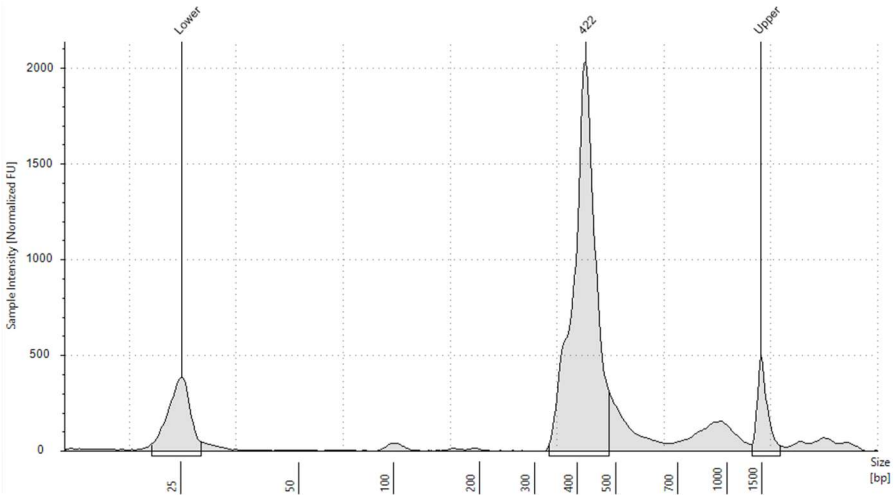
Note: The quantification method may be adapted to an in-house available and/or preferred method, such as qPCR. When quantifying using TapeStation or Bioanalyzer, we recommend to reduce the initial loading concentration of the library on the sequencer to avoid overclustering.

4.2. **Optional but recommended:** Perform a qualitative verification of the libraries on TapeStation or Bioanalyzer, according to the manufacturer's protocol. If needed, dilute the pool. E.g. for TapeStation High Sensitivity kit, dilute to ~2 ng/µL.

Example of clean libraries on TapeStation:
Panel A



Panel B

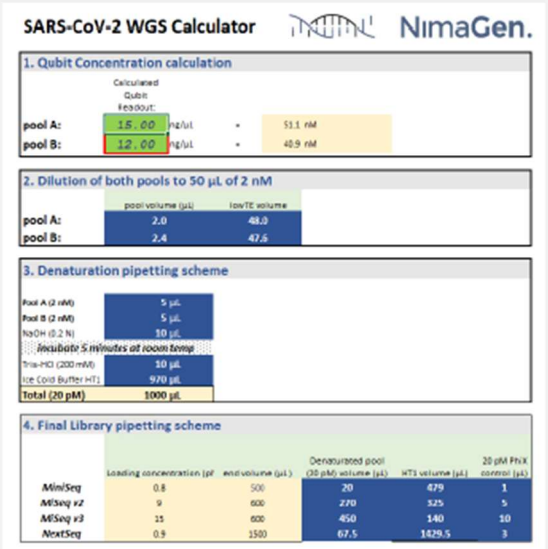


4.3. Perform sequencing on an illumina® platform, according to the manufacturer's manual.

Highly recommended: Use the dedicated SARS-CoV-2 WGS calculator at the download section on the website.
Otherwise, use the sequencing guidelines below.

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 ng/µL. 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).



a. We recommend a length of 445 bp for calculating the library molarity for panel A and 445 bp for panel B.
b. Combine equimolar solutions of panel A and B in a ratio of 1:1 prior to denaturing.
c. We advise to maintain a minimal read depth per sample of 150K-200K clusters.
d. A spike-in of 5% PhiX is recommended/required for QC purposes.

e. We advise to start with a lower loading concentration for the initial sequence run and adjust for subsequent runs if needed. This avoids overclustering and potential failure of the run. See table 1 for sequencing guidelines.

Table 1 Illumina sequencer and sample multiplexing guidelines

Sequencer	Reagent kit	Run setup	Library concentration
MiniSeq	Mid/High output 300 cycles	151-8-8-151	0.8 pM
NextSeq	300 cycles	151-8-8-151	0.9 pM
MiSeq	V2 300 cycles	151-10-10-151	9 pM
MiSeq	V3 300 cycles	151-10-10-151	15 pM

Data Analysis

Software tools:
JSI – Virseak

QIAGEN - CLC Bio

Ridom – SeqSphere

Open Source GITHUB
Co-development Nimagen & Radboud University Medical Center
Department of Medical Microbiology - Dr Jordy Coolen

APPENDIX 1

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).

Quick Start Protocol for generation of 11 µL cDNA:

- Lunascript Supermix 2.2 µL
- Water 3.3 µL
- RNA sample 5.5 µL
- Total 11 µL

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.

Customer Support

For technical questions, assistance, or to suggest enhancements, please contact us at techsupport@nimagen.com.

Revision History

Section	Summary of changes	Version	Date
All	Miseq v2 loading changed from 5.5 to 9pM MiSeq v3 loading changed from 10 to 15pM EBT buffer → LowTE	V1.6	2021-01-19
Sequencing section	iSeq100 kit Safe stopping points added	V1.7	2021-01-20
All	Reverse Transcription kit recommendation, safe stopping points info, tube labelling	V1.8	2021-02-19
All	safe stopping points info, tube labelling	V1..9	2021-03-02
Reaction set-up	Naming of Probe Dilution Buffer Plus Thaw, mix and centrifuge of reagents before use	V1.10	2021-04-02
All	Vortexing of complete 20 µL RCPCR mixture prior to thermo-profiling. Updated picture of library calculator Addition of index plate UDI04D Addition of Lunascript protocol	V2.01	2021-04-09
All	Probe panel update	V3.01	2021-10-27
All	For use with SARS-CoV-2 WGS kits of version 4.0 or higher Probe Panel update to optimize for Omicron variant (bed files not changed) IDX plates dried down Amendment to thermal protocol	V4.0	2021-01-16
All	Layout, order and formulation changed	V5.0	2024-01-24
Kit Contents Required Material	Change of kit components MM and PDB Small adjustments in the required materials.	V6.0	2026-01-23

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)

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