NimaGen.

EasySeq™

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

NGS library prep by Reverse Complement PCR

Version: RC-COVID096-v1.9
Revision date: March 2, 2021

Product and Company Information

Product name:	EasySeq™ SARS-CoV-2 Whole Genome NGS Sequencing kit
Product Info:	www.nimagen.com/covid19
Product use:	Research Use Only

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Product Use

Multiplex Amplicon based NGS Library preparation for sequencing the genome of SARS-CoV-2 virus. To be used to detect mutations, monitoring viral populations for epidemiology and outbreak events. This Reverse-Complement PCR based library prep contains all reagents to generate Illumina® compatible libraries in a simple, sensitive and robust method for fast and cost-effective WGS of the viral genome.

Kit Content.

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

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 mix (blue cap)	1 (Frozen)	Tube 500 µL
AmpliClean Bead solution*	2 (Cooled)	Tube 1.7 mL
RC-PCR Low-TE Buffer (yellow cap)	2 (Cooled)	Tubes 2 x 1100 µL

NimaGen Part# MMHS-25		Content
PCR 2x Hotstart HiFi Mastermix, compatible with RC-PCR (white cap). p/n MMHS-25	1 (Frozen)	Tubes 2 x 1100 µL

Choose one of the index plates of choice

NimaGen Part# IDX96-U0xD		Content
2 x IDX Index Primer Plates*. Choose one of the 5 available Index Plate (U01D, U02D, U03D, U05D, U06D) with 96 Unique Dual, 10 bp Indexes for Illumina®. p/n IDX96-U0xD	1 (Frozen)	2 x 96-well plates (pre-spotted with 4 µL Index Primers per well)

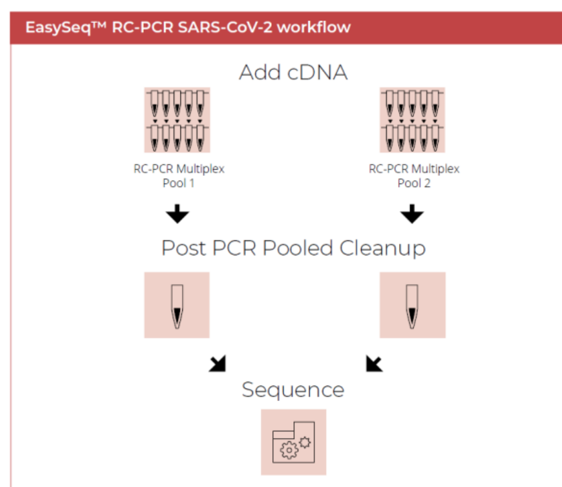
**IDX plates are semi-skirted, ABI stye PCR Plates, dividable per 24 rxns, containing 4 µL of Unique Dual Index primer pairs in each well, ready to use. Index Sequences can be downloaded from the product page, section "downloads"*

Needed, but not included

Description	Vendor
Adjustable Pipette Set (P10, P20, P200, P1000)	Multiple Vendors
LunaScript (p/n E3010L) cDNA synthesis kit (download protocol)	New England Biolabs
Agarose Gel system, TapeStation, Bioanalyzer Instrument, or equivalent, incl. consumables	Agilent® or other
Ethanol absolute, mol. biol. grade	Multiple Vendors
General plasticware, DNAse free (1.5mL tubes, pipette tips w/filter)	Multiple Vendors
Ice or tabletop cooling block	Multiple Vendors
Illumina® NGS Sequencing instrument (iSeq, MiSeq, MiniSeq, NextSeq)	Illumina®
Illumina® Sequencing Reagent kit (min. 2x150 bp run)	Illumina®
Mini Spinner for 1.5 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®
Thermocycler 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

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



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	1 minute	Max	
98°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	1 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 cycler.

2. Reverse Complement PCR

The target specific RC-probes will be transformed into the functional, tailed and indexed PCR primers, followed by multiplex cDNA amplification of the target regions. Click here for example protocols 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 (Blue cap)
- PCR 2x Hotstart HiFi Mastermix (White cap)

Note: The 2x mastermix contains isostabilizers and may not freeze completely, even when stored at -15°C to -25°C. The mastermix 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 IDX PCR plates and 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 https://www.nimagen.com/covid19

Note: For each sample, two PCR reactions are needed (pool A and pool B). Use always the same strip and well position for the same sample, in order to have identical indexes for the sample in both pools. Example: For setting up 24 samples, cut off strip 1-3 from both IDX plates and store the remaining of the plates immediately at -20°C.

2.3. Prepare in a fresh 1.5 mL Eppendorf tube the 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 per reaction (blue cap)
- 10 µL RC-PCR 2x mastermix per reaction (white cap)

Prepare in a fresh 1.5 mL Eppendorf tube the Probe-Polymerase premix B, by combining and mixing:

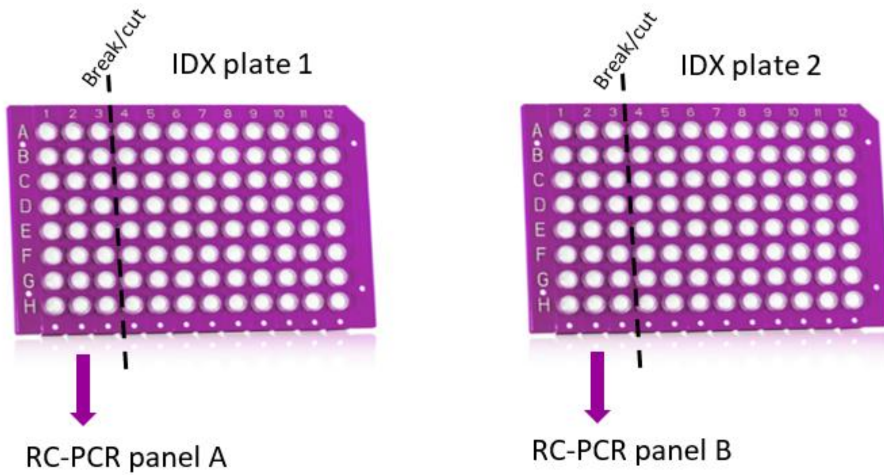
- 0.2 µL panel **B** probe mix per reaction (red cap)
- 0.8 µL RC-PCR probe dilution buffer per reaction (blue cap)
- 10 µL RC-PCR 2x mastermix per reaction (white cap)

Example: 48 samples + 10% extra volume*

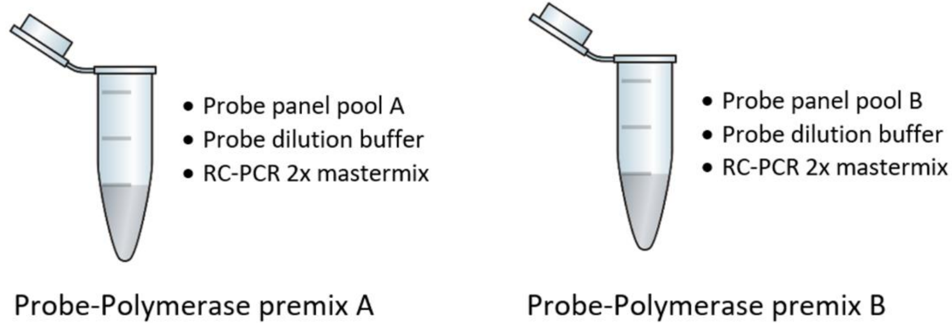
- **Tube A:**
  - **10.56 µL Panel A probe mix (black cap)**
  - **42.24 µL RC-PCR probe dilution buffer (blue cap)**
  - **528 µL RC-PCR 2x mastermix (white cap)**
- **Tube B:**
  - **10.56 µL Panel A probe mix (red cap)**
  - **42.24 µL RC-PCR probe dilution buffer (blue cap)**
  - **528 µL RC-PCR 2x mastermix (white cap)**

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

Step 2.2 Take the two IDX PCR plates and cut off the number of strips needed.



Step 2.3 Prepare in two 1.5 mL eppendorf tubes the Probe-Polymerase premix



2.4. Quick-spin the PCR plates (A and B), and remove seals carefully.

2.5. Add to each tube of plate A: 11 µL of the Probe-Polymerase premix A.

2.6. Add to each tube of plate B: 11 µL of the Probe-Polymerase premix B.

2.7. Add to the same well position of both plate/strips A and B: 5 µL cDNA.

Example: Add 5 µL of cDNA from patient 1 to both wells A1 of plate A and B → total cDNA per sample needed is 10 µL.

2.8. Close the tube strips carefully with caps (included in the kit) and mix by flicking.

2.9. Short spin.

2.10. Start the RC-PCR program in the thermal cycler(s) and place samples in the cycler when the block is between 60°C and 98°C, close the lid.

Note: When running >48 samples in a run, two PCR cyclers are needed.

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

The samples have now been amplified and tagged with sample specific indexes and sequencing tails. From this point, PCR products will be pooled together in two tubes, as their corresponding pool A and B, purified by a "2*x*" 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 recommended 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 (Brown Cap) to Room Temperature.

3.2. Mark 2 x 1.5 mL Eppendorf tubes with resp. "A" and "B".

3.3. Create, in the tubes A and B, two pools, by combining RC-PCR products from all the wells in each of the two plates (except negative controls), 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.
Note: If Ct values are unknow, pool 5 µL of each PCR product.

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™ beads purification:

Purification #1

a. Vortex beads thoroughly to resuspend.
b. Add 85 µL beads solution to the two 100 µL 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.

2x Ethanol wash

e. Remove and discard liquid carefully without disturbing the beads.
f. Add 200 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. (Optionally a quick spin can be performed, then place tube back on magnet and remove excess ethanol)
j. Dry with open cap for 2-3 minutes at Room Temperature. **Do not over-dry.**
k. On Magnet: Add 110 µL 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 µL 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 µL resuspended beads solution to the two 100 µL 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.

2x Ethanol wash

s. Remove and discard liquid carefully without disturbing the beads.
t. Add 200 µL (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 excess ethanol)
x. Dry with open cap for 2-3 minutes at Room Temperature. **Do not over-dry.**

3.7. Elution

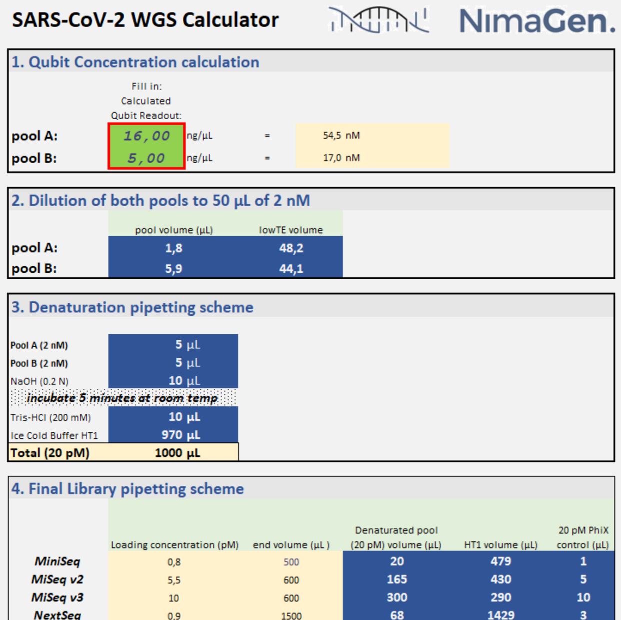
a. On Magnet: Add 50 µL RC-PCR Low TE buffer to the two tubes A3 and B3
b. Off Magnet: Re-suspend the beads by flicking or short vortexing.
c. Incubate for 2 minutes, off magnet.
d. Put on magnet and wait for 3-5 minutes or for the solutions to be fully cleared.
e. Carefully bring 40 µL of the clear 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°C if not continuing same day.

Libraries are now ready for quantification and qualification.

3.6 Determine final concentration of both A4 and B4 by a duplo Qubit (HS) measurement according to manufacturer's manual. Use the dedicated SARS-CoV-2 WGS calculator at the download section of www.nimagen.com/covid19

Step 3.6: 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).



SARS-CoV-2 WGS Calculator NimaGen.

1. Qubit Concentration calculation

	Fill in: Calculated Qubit Readout:			
pool A:	16,00	ng/µL	=	54,5 nM
pool B:	5,00	ng/µL	=	17,0 nM

2. Dilution of both pools to 50 µL of 2 nM

	pool volume (µL)	lowTE volume
pool A:	1,8	48,2
pool B:	5,9	44,1

3. Denaturation pipetting scheme

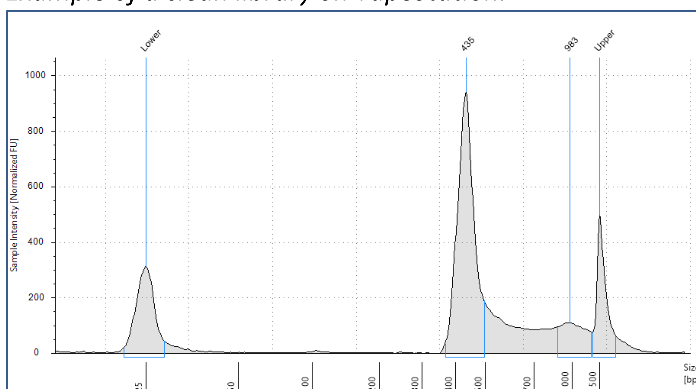
Pool A (2 nM)	5 µL
Pool B (2 nM)	5 µL
NaOH (0.2 N)	10 µL
incubate 5 minutes at room temp	
Tris-HCl (200 mM)	10 µL
Ice Cold Buffer HT1	970 µL
Total (20 pM)	**1000 µL**

4. Final Library pipetting scheme

	Loading concentration (pM)	end volume (µL)	Denaturated pool (20 pM) volume (µL)	HT1 volume (µL)	20 pM PhiX control (µL)
MiniSeq	0,8	500	20	479	1
MiSeq v2	5,5	600	165	430	5
MiSeq v3	10	600	300	290	10
NextSeq	0,9	1500	68	1429	3

3.7 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:



3.8 Perform Sequencing on an Illumina® NGS platform, according to the manufacturer's manual. Appendix 1 outlines the detailed Illumina® NGS protocols.

NOTE: Download Index Sequences from www.nimagen.com/covid19, section "downloads" to create a sample sheet. For technical assistance contact our technical support at techsupport@nimagen.com.

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	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	D: iSeq100 kit Save 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

Reference:

Wolters et al., 2020: “Novel SARS-CoV-2 Whole-genome sequencing technique using Reverse Complement PCR enables fast and accurate outbreak analysis”.
doi: https://doi.org/10.1101/2020.10.29.360578

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