applied biosystems

PROTOCOL

Sanger sequencing

Protocol for Sanger sequencing of the SARS-CoV-2 spike (S) gene

SARS-CoV-2 infections continue to be a challenge across the globe. Part of the challenge, often seen with viruses, is that the nucleic acid genome quickly mutates, producing new strain lineages. These new lineages may spread more quickly, cause either milder or more severe disease, may have decreased susceptibility to therapeutic agents, and may evade vaccine-induced immunity. Importantly, they can also have the ability to evade detection by sequence-based diagnostic tests, complicating epidemiological monitoring. While the SARS-CoV-2 mutation rate is thought to be lower than those of other RNA viruses, the sheer number of infections raises the chances that novel strain lineages will appear in circulation [1]. Recently, two new lineages that appear to have increased infectivity have been identified [2,3]. Interestingly, both of these lineages have many new mutations in the SARS-CoV-2 spike (S) gene. Because mutations in the S gene have the potential to affect interactions with the angiotensin-converting enzyme 2 (ACE2) receptor [4], it is important to monitor S gene sequences for new mutations.

We therefore developed a protocol for analyzing the entire S gene by Sanger sequencing. The primer sequences used here are based on those published by the Centers for Disease Control and Prevention (CDC) [5]. Briefly, cDNA synthesis is performed on a sample containing viral RNA. Next, the cDNA is used in specific regions of target amplification using tailed primers that cover the S gene. For this, the Applied Biosystems™ BigDye™ Direct Cycle Sequencing Kit and M13 sequence–tagged primer sets are used. The amplified sequences are then subjected to cycle sequencing using either M13-forward or M13-reverse primers provided in the BigDye Direct Cycle Sequencing Kit. Unincorporated nucleotides and primers are next removed using the Applied Biosystems™ BigDye XTerminator™ Purification Kit, and the sequences are read by standard capillary electrophoresis (CE). The sequences obtained can be read by any sequencing program, such as SeqA or Geneious™ software, and compared with known or expected SARS-CoV-2 sequences (Figure 1).

Some of the sequences generated by this method will produce CE traces that may be difficult to interpret. To determine whether a sequencing trace was useful, we employed quality control metrics generated by Applied Biosystems™ Sequence Scanner Software v2.0. These metrics include trace score (average of basecaller quality values for bases in the clear range), contiguous read length (CRL), and QV20+ (total number of bases in the entire trace that have a basecaller quality value of ≥20). Guidelines for using these metrics for QC and analysis of results are given at the end of the protocol. However, standard analysis of sequencing traces is often sufficient to determine whether a novel sequence is present.

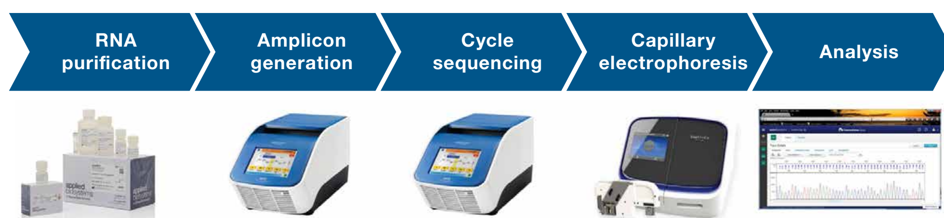

Figure 1. Workflow detection of new SARS-CoV-2 lineages using Sanger sequencing. RNA is purified from samples using standard techniques. cDNA is synthesized from the RNA, and specific M13 sequence–tagged amplicons are generated by PCR. The amplicons are sequenced in the forward and reverse directions using universal M13 primers and the BigDye Direct Cycle Sequencing Kit. The sequencing reactions are cleaned using the BigDye XTerminator kit and subjected to CE. The resulting sequencing traces can be analyzed and compared to reference SARS-CoV-2 sequences to determine if the lineages are present.

ThermoFisher SCIENTIFIC

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

IMPORTANT: This protocol is very sensitive; therefore, utmost care must be taken to prepare the stock solutions and set up the amplification reactions in an amplicon-free environment.

1. Materials needed

1.1 Equipment

Product	Supplier	Cat. No.
Veriti 96-Well Fast Thermal Cycler, ProFlex 96-Well PCR System, or similar thermal cycler	Thermo Fisher Scientific	4375305 or 4484075
MicroMixer E-36 for 96-well plates	Taitec	0027765-000
Single-channel and multichannel micropipettes of various sizes capable of pipetting volumes from 1.00 µL to 1,000.0 µL	MLS	Any
Cold block or ice	MLS	Any
Plate centrifuge	MLS	Any
Microcentrifuge or mini centrifuge	MLS	Any
Vortex mixer	MLS	Any

1.2 Reagents, kits, and consumables

Product	Supplier	Cat. No.
SuperScript IV VILO Master Mix	Thermo Fisher Scientific	117565500
Nuclease-Free Water	Thermo Fisher Scientific	AM9937 or equivalent
BigDye Direct Cycle Sequencing Kit	Thermo Fisher Scientific	4458688 or equivalent
BigDye XTerminator Purification Kit	Thermo Fisher Scientific	4376486 or equivalent
MicroAmp Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	Thermo Fisher Scientific	4346906 or 4366932
MicroAmp Optical Adhesive Film	Thermo Fisher Scientific	4311971, 4313663, or 4360954
Nonstick, RNase-Free Microcentrifuge Tubes, 1.5 mL	Thermo Fisher Scientific	AM12450 or equivalent
5 mL tube, PCR clean	MLS	Any
Sterilized aerosol barrier (filter) pipette tips	MLS	Any

1.3 Primers

- Primer sequences are given in Table 1.

Table 1. Sequences of M13-tagged primers for analyzing the S gene. A subset of primer pairs that focus on specific regions of the S gene can be chosen according to researchers' needs; the complete list is provided in here. The M13 sequence tags are highlighted in red.

Coordinates*	Forward primer name	Forward primer sequence	Reverse primer name	Reverse primer sequence
20990-21562	SC2M1-54_LEFT_M13	TGTAAAACGACGGCCAGTTGATTGGTGATTGTGCAACTGTACA	SC2M1-54_RIGHT_M13	CAGGAAACAGCTATGACCTGTTCGTTTAGTTGTTAACAAGAACATCA
21421-21916	SC2M1-55_LEFT_M13	TGTAAAACGACGGCCAGTAGGGGTACTGCTGTTATGTCTTTAAA	SC2M1-55_RIGHT_M13	CAGGAAACAGCTATGACCAAGTAGGGACTGGGTCTTCGAA
21775-22345	SC2M1-56_LEFT_M13	TGTAAAACGACGGCCAGTTGGGACCAATGGTACTAAGAGGT	SC2M1-56_RIGHT_M13	CAGGAAACAGCTATGACCACCAGCTGTCCAACCTGAAGAA
22203-22697	SC2M1-57_LEFT_M13	TGTAAAACGACGGCCAGTGTGATCTCCCTCAGGGTTTTTCG	SC2M1-57_RIGHT_M13	CAGGAAACAGCTATGACCACTTAAAAGTGGAAAATGATGCGGAA
22563-23128	SC2M1-58_LEFT_M13	TGTAAAACGACGGCCAGTACTTGTGCCCTTTTGGTGAAGT	SC2M1-58_RIGHT_M13	CAGGAAACAGCTATGACCTGCTGGTGCATGTAGAAGTTCA
22986-23519	SC2M1-59_LEFT_M13	TGTAAAACGACGGCCAGTCCGGTAGCACACCTTGTAATGG	SC2M1-59_RIGHT_M13	CAGGAAACAGCTATGACCCCCCTATTAAACAGCCTGCACG
23379-23876	SC2M1-60_LEFT_M13	TGTAAAACGACGGCCAGTACCAGGTTGCTGTTCTTTATCAGG	SC2M1-60_RIGHT_M13	CAGGAAACAGCTATGACCCAGCTATTCCAGTTAAAGCACGGT
23737-24231	SC2M1-61_LEFT_M13	TGTAAAACGACGGCCAGTAATTCTACCAGTGTCTATGACCAAGAC	SC2M1-61_RIGHT_M13	CAGGAAACAGCTATGACCGCACCAAAGGTCCAACCAGAAG
24095-24623	SC2M1-62b_LEFT_M13	TGTAAAACGACGGCCAGTTGCAGATGCTGGCTTCATCA	SC2M1-62b_RIGHT_M13	CAGGAAACAGCTATGACCCACACTCTGACATTTTAGTAGCAGC
24493-25003	SC2M1-63_LEFT_M13	TGTAAAACGACGGCCAGTAAATGATATCCTTTCACGTCTTGACAAA	SC2M1-63_RIGHT_M13	CAGGAAACAGCTATGACCTGAGTCTAATTCAGGTTGCAAAGGA
24858-25369	SC2M1-64_LEFT_M13	TGTAAAACGACGGCCAGTGCACACACTGGTTTGTAACACAA	SC2M1-64_RIGHT_M13	CAGGAAACAGCTATGACCTTTGACTCCTTTGAGCACTGGC
25214-25790	SC2M1-65_LEFT_M13	TGTAAAACGACGGCCAGTTAGGTTTTATAGCTGGCTTGATTGC	SC2M1-65_RIGHT_M13	CAGGAAACAGCTATGACCCATTTCCAGCAAAGCCAAAGCC

* Based on NC_045512.2 coordinates.

- Primers can be ordered from our **custom oligo ordering web page** (**https://www.thermofisher.com/order/custom-standard-oligo**).
  - 25 nmol of dried and desalted primers can be ordered, but order can be scaled up as needed.
- Resuspend dried oligos to final concentration of 100 µM with TE buffer.

1.4. Amplification mixes of primers

- Prepare the target-specific amplification primer mixes:
  - Label clean microcentrifuge tubes for each primer pair (e.g., SC2M1-54, SC2M1-55, etc.). Add 492 µL of TE buffer to each tube.
  - Add 4 µL each of both the left and right oligos of a pair to the appropriate tube (i.e., SC2M1-54_LEFT_M13 and SC2M1-54_RIGHT_M13 in one tube, SC2M1-55_LEFT_M13 and SC2M1-55_RIGHT_M13 to the next, etc.).
  - These will be the 10X sequencing amplification primer mixes, with each oligo at 0.8 µM, that will be used in step 3.1–3.2.

2. cDNA synthesis

2.1. For each sample, combine:

Reagent	Final volume 50 µL
5X SuperScript IV VILO Master Mix	10 µL
Sample	1–15 µL
Water	To final 50 µL

2.2. Vortex for 2–3 seconds, then centrifuge briefly (5–10 seconds) at 1,000 x *g*.
Note: Sample input volume can be adjusted for sensitivity. For example, up to 15 µL of a sample that is expected to have low titer may be used.

2.3. Reverse transcription

2.3.1. Program a thermal cycler with the following profile:

Parameter	Stage/step			
	Annealing	Polymerase extension	Polymerase inactivation	Hold
Temperature	25°C	50°C	80°C	4°C
Time	10 min	15 min	10 min	Indefinitely

2.3.2. Put samples in the thermal cycler and run the program.
Note: Samples can be held at 4°C or on ice for up to 8 hours; for longer storage, freeze at –20°C.

3. PCR amplifications of targets

3.1. For each sample, a forward and reverse reaction will be run. The initial PCR amplification, therefore, requires two identical reactions to be set up. An example 96-well plate setup for four samples is shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	SC2M1-54 primers	SC2M1-54 primers	SC2M1-55 primers	SC2M1-55 primers	SC2M1-56 primers	SC2M1-56 primers	SC2M1-57 primers	SC2M1-57 primers	SC2M1-58 primers	SC2M1-58 primers	SC2M1-59 primers	SC2M1-59 primers
B	SC2M1-60 primers	SC2M1-60 primers	SC2M1-61 primers	SC2M1-61 primers	SC2M1-62b primers	SC2M1-62b primers	SC2M1-63 primers	SC2M1-63 primers	SC2M1-64 primers	SC2M1-64 primers	SC2M1-65 primers	SC2M1-65 primers
C	SC2M1-54 primers	SC2M1-54 primers	SC2M1-55 primers	SC2M1-55 primers	SC2M1-56 primers	SC2M1-56 primers	SC2M1-57 primers	SC2M1-57 primers	SC2M1-58 primers	SC2M1-58 primers	SC2M1-59 primers	SC2M1-59 primers
D	SC2M1-60 primers	SC2M1-60 primers	SC2M1-61 primers	SC2M1-61 primers	SC2M1-62b primers	SC2M1-62b primers	SC2M1-63 primers	SC2M1-63 primers	SC2M1-64 primers	SC2M1-64 primers	SC2M1-65 primers	SC2M1-65 primers
E	SC2M1-54 primers	SC2M1-54 primers	SC2M1-55 primers	SC2M1-55 primers	SC2M1-56 primers	SC2M1-56 primers	SC2M1-57 primers	SC2M1-57 primers	SC2M1-58 primers	SC2M1-58 primers	SC2M1-59 primers	SC2M1-59 primers
F	SC2M1-60 primers	SC2M1-60 primers	SC2M1-61 primers	SC2M1-61 primers	SC2M1-62b primers	SC2M1-62b primers	SC2M1-63 primers	SC2M1-63 primers	SC2M1-64 primers	SC2M1-64 primers	SC2M1-65 primers	SC2M1-65 primers
G	SC2M1-54 primers	SC2M1-54 primers	SC2M1-55 primers	SC2M1-55 primers	SC2M1-56 primers	SC2M1-56 primers	SC2M1-57 primers	SC2M1-57 primers	SC2M1-58 primers	SC2M1-58 primers	SC2M1-59 primers	SC2M1-59 primers
H	SC2M1-60 primers	SC2M1-60 primers	SC2M1-61 primers	SC2M1-61 primers	SC2M1-62b primers	SC2M1-62b primers	SC2M1-63 primers	SC2M1-63 primers	SC2M1-64 primers	SC2M1-64 primers	SC2M1-65 primers	SC2M1-65 primers

Note: Reactions using the same cDNA sample have identical color coding.

Note: The layout above is for querying the entire S gene. If only a subset of amplicons is to be analyzed, the layout can be adjusted accordingly.

Note: Positive and negative control samples can be run on the same or a different plate; the negative control is a no-template control (NTC).

3.2. In each well of a 96-well PCR plate, combine:

- 1.5 µL of 10X sequencing amplification primer mix in duplicate (as suggested in the table above)
- 5 µL of 2X BigDye Direct PCR Master Mix (supplied in kit)
- 1 µL of cDNA sample from completed step 2.3
  - Leftover cDNA sample can be frozen at –20°C.
- Water to 10 µL total volume

3.3. Seal the plate; vortex for 2–3 seconds, then centrifuge briefly (5–10 seconds) at 1,000 x *g*.

3.4. Place the plate into a thermal cycler and run the following program:

Parameter	Stage/step				
	Polymerase activation	Cycling (40 cycles)			Hold
		Denaturation	Annealing	Extension	
Temperature	95°C	96°C	62°C	68°C	4°C
Time	10 min	3 sec	15 sec	30 sec	Indefinitely

Note: Samples can be held at 4°C or on ice for up to 8 hours; for longer storage, freeze at –20°C.

Note: Do not exceed 10 µL total reaction volume. The cycle sequencing and BigDye Xterminator steps have been optimized for 10 µL input volumes.

4. Cycle sequencing

4.1. Once the PCR in step 3.4 is complete, the plate can be used directly for cycle sequencing.

4.2. Remove the seal from the plate.

4.3. To each well of the plate, add:

- 2 µL of BigDye Direct Sequencing Master Mix (supplied in kit)
- 1 µL of BigDye Direct M13 Forward or M13 Reverse primer (supplied in kit)

Note: It is important to add the M13 Forward primer to one of the duplicate PCR reactions, and the M13 Reverse primer to the other reaction. An example based on the plate setup is shown below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	SC2M1-54 M13 For	SC2M1-54 M13 Rev	SC2M1-55 M13 For	SC2M1-55 M13 Rev	SC2M1-56 M13 For	SC2M1-56 M13 Rev	SC2M1-57 M13 For	SC2M1-57 M13 Rev	SC2M1-58 M13 For	SC2M1-58 M13 Rev	SC2M1-59 M13 For	SC2M1-59 M13 Rev
B	SC2M1-60 M13 For	SC2M1-60 M13 Rev	SC2M1-61 M13 For	SC2M1-61 M13 Rev	SC2M1-62b M13 For	SC2M1-62b M13 Rev	SC2M1-63 M13 For	SC2M1-63 M13 Rev	SC2M1-64 M13 For	SC2M1-64 M13 Rev	SC2M1-65 M13 For	SC2M1-65 M13 Rev
C	SC2M1-54 M13 For	SC2M1-54 M13 Rev	SC2M1-55 M13 For	SC2M1-55 M13 Rev	SC2M1-56 M13 For	SC2M1-56 M13 Rev	SC2M1-57 M13 For	SC2M1-57 M13 Rev	SC2M1-58 M13 For	SC2M1-58 M13 Rev	SC2M1-59 M13 For	SC2M1-59 M13 Rev
D	SC2M1-60 M13 For	SC2M1-60 M13 Rev	SC2M1-61 M13 For	SC2M1-61 M13 Rev	SC2M1-62b M13 For	SC2M1-62b M13 Rev	SC2M1-63 M13 For	SC2M1-63 M13 Rev	SC2M1-64 M13 For	SC2M1-64 M13 Rev	SC2M1-65 M13 For	SC2M1-65 M13 Rev
E	SC2M1-54 M13 For	SC2M1-54 M13 Rev	SC2M1-55 M13 For	SC2M1-55 M13 Rev	SC2M1-56 M13 For	SC2M1-56 M13 Rev	SC2M1-57 M13 For	SC2M1-57 M13 Rev	SC2M1-58 M13 For	SC2M1-58 M13 Rev	SC2M1-59 M13 For	SC2M1-59 M13 Rev
F	SC2M1-60 M13 For	SC2M1-60 M13 Rev	SC2M1-61 M13 For	SC2M1-61 M13 Rev	SC2M1-62b M13 For	SC2M1-62b M13 Rev	SC2M1-63 M13 For	SC2M1-63 M13 Rev	SC2M1-64 M13 For	SC2M1-64 M13 Rev	SC2M1-65 M13 For	SC2M1-65 M13 Rev
G	SC2M1-54 M13 For	SC2M1-54 M13 Rev	SC2M1-55 M13 For	SC2M1-55 M13 Rev	SC2M1-56 M13 For	SC2M1-56 M13 Rev	SC2M1-57 M13 For	SC2M1-57 M13 Rev	SC2M1-58 M13 For	SC2M1-58 M13 Rev	SC2M1-59 M13 For	SC2M1-59 M13 Rev
H	SC2M1-60 M13 For	SC2M1-60 M13 Rev	SC2M1-61 M13 For	SC2M1-61 M13 Rev	SC2M1-62b M13 For	SC2M1-62b M13 Rev	SC2M1-63 M13 For	SC2M1-63 M13 Rev	SC2M1-64 M13 For	SC2M1-64 M13 Rev	SC2M1-65 M13 For	SC2M1-65 M13 Rev

4.4. Seal the plate. Vortex for 2–3 seconds, then centrifuge briefly (5–10 seconds) at 1,000 x *g*.

4.5. Place the plate into a thermal cycler and run the following program:

	Stage/step						
				Cycling (25 cycles)			
Parameter	**Post PCR cleanup**	**Post PCR inactivation**	**Polymerase activation**	**Denaturation**	**Annealing**	**Extension**	**Hold**
Temperature	37°C	80°C	96°C	96°C	50°C	60°C	4°C
Time	15 min	2 min	1 min	10 sec	5 sec	75 sec	Indefinitely

5. Sequencing cleanup

5.1. Spin the reaction plate at 1,000 x *g* for 1 minute, then remove the seal.

5.2. Prepare a mix with SAM Solution and BigDye XTerminator™ Solution in an appropriately sized tube.

5.2.1. Calculate the amount of SAM Solution and XTerminator Solution needed for all samples. You will need 45 µL of SAM Solution and 10 µL of XTerminator Solution per well.

5.2.2. Add the calculated volume of SAM Solution to a new tube using a conventional pipette tip.

Note: Make sure there are no particulates in the SAM Solution before pipetting. If there are particulates, heat the SAM Solution to 37°C and mix to dissolve. Cool to room temperature before using.

5.2.3. Vortex the bulk container of XTerminator Solution at maximum speed for at least 10 seconds, until the solution is homogeneous.

5.2.4. Using a wide-bore pipette tip, add the calculated volume of XTerminator Solution to the tube.

IMPORTANT: Avoid pipetting from the top of the liquid.

5.2.5. Mix the tube of combined reagents until homogeneous.

5.3. Add 55 µL of the SAM Solution/XTerminator Solution mix to each well.

IMPORTANT: Avoid pipetting from the top of the liquid. When aliquoting into the plate, re-vortex the SAM Solution/XTerminator Solution mix every 8–10 wells to homogenize the bead mixture.

5.4. Seal the plate with Applied Biosystems™ MicroAmp™ Optical Adhesive Film. Make sure the plate is sealed well.

5.5. Vortex the reaction plate for 40 minutes.

5.6. In a swinging-bucket centrifuge, spin the plate at 1,000 x *g* for 2 minutes.

6. Collect data

6.1. Make sure the instrument is calibrated with the correct sequencing standard (Z-dye set matrix and sequencing standard)

- For details, see the Applied Biosystems™ 3500/3500xL Genetic Analyzer User Guide or SeqStudio™ Genetic Analyzer Getting Started Guide.

6.2. Remove the MicroAmp film and replace it with a 96-well plate septum.

6.3. Load plates into the genetic analyzer.

6.4. Select or create an appropriate run module according to your capillary length, number of capillaries, and polymer type on your instrument. The recommended default run modules are listed below:

- For 3500xL instruments with 50 cm capillaries:
  - Instrument protocol: BDxFastSeq50_POP7xl_Z

    Note: Replace 50 with 36 in the instrument protocol name if you have a 36 cm capillary installed.
  - Analysis Module: BDTv3.1_PA_Protocol-POP7
- For SeqStudio instruments:
  - MedSeqBDX

7. Analyze results using a sequencing program

Sequence Scanner v2.0 is free software for viewing electropherograms. It provides an easy way to perform a high-level sequencing data quality check or general data review that includes summary tables and electropherograms as well as a general raw or analyzed data view for .ab1 files.

7.1. To obtain the software, go to **thermofisher.com/pages/WE28396/**

7.2. Using Sequence Scanner Software v2.0, generate a QC report. For each sequencing trace, determine the trace score, CRL, and the QV20+ score.

7.3. Suggested acceptance criteria:

- A sequencing trace is acceptable as positive if two of the three thresholds are met:
  - Trace score greater than 31
  - CRL greater than 50
  - QV20+ greater than 50
- A sequencing trace is acceptable as negative if two of the three thresholds are met:
  - Trace score less than 14
  - CRL less than 24
  - QV20+ less than 24
- Sequencing traces that do not fit the above criteria are indeterminate and should be repeated

7.4 Using BLAST™ alignment or another sequence alignment tool, align positive traces to the SARS-CoV-2 genome.

- Alignments greater than 85% over read length are considered homologous to the SARS-CoV-2 genome
- Discard any sequences that are not homologous to SARS-CoV-2

7.5 For variant analysis in any of the amplicons, these criteria should be met:

- Positive (passable) traces in both directions (7.3)
- Homology to the SARS-CoV-2 genome (7.4) in regions outside the putative variant
- Negative traces in NTC reactions (7.3)

7.6. Test runs that fail for reasons not attributable to system performance, such as equipment malfunction, operator error, or other demonstrable cause, will be designated as invalid runs. Invalid runs will be retested and documented in the study report(s).

applied
biosystems

References

1. van Dorp L et al.(2020) No evidence for increased transmissibility from recurrent mutations in SARS-CoV-2. *Nat Commun* 11, 5986. **https://doi.org/10.1038/s41467-020-19818-2**
2. Wise J (2020) Covid-19: New coronavirus variant is identified in UK. *BMJ* Dec 16;371:m4857. **doi: 10.1136/bmj.m4857**
3. Tegally H et al. (2020) Emergence and rapid spread of a new severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) lineage with multiple spike mutations in South Africa. *medRxiv* **https://doi.org/10.1101/2020.12.21.20248640**
4. Conceicao C et al. (2020) The SARS-CoV-2 Spike protein has a broad tropism for mammalian ACE2 proteins. *PLoS Bio* 18(12):e3001016. **https://doi.org/10.1371/journal.pbio.3001016**
5. Paden CR et al. (2020) Rapid, sensitive, full-genome sequencing of severe acute respiratory syndrome coronavirus 2. *Emerg Infect Dis.* 26(10):2401-2405. **doi.org/10.3201/eid2610.201800**

Find out more at **thermofisher.com/sangercoronavirus**

ThermoFisher
SCIENTIFIC

For Research Use Only. Not for use in diagnostic procedures. © 2021 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified. BLAST is a trademark of the National Liberty of Medicine. **COL34063 0321**