

Protocol for sequencing the SARS-CoV-2 S gene 69-70del

The global emergency of SARS-CoV-2 infections has caused significant disruptions to economic and personal activities and poses a serious health risk. Recently, a new variant that appears to have increased infectivity was identified in populations in England [1]. Interestingly, this variant was also associated with a high rate of failure to detect one of the genes when PCR tests were employed. Investigators observed that one of the mutations in the spike (S) gene, which deletes amino acids 69 and 70 (S gene 69-70del), caused a reproducible failure to detect S genes by PCR, although the other viral genes were detectable [2-3]. It is therefore important to determine whether samples with S gene PCR dropouts have sequence changes that interfere with the generation of the PCR results.

We developed a protocol for analyzing SARS-CoV-2 S gene sequences by Sanger sequencing (Figure 1). This protocol has been developed to work with purified RNA samples currently being analyzed with the Applied Biosystems™ TaqPath™ COVID-19 Combo Kit. To meet the low limit of detection associated with this kit, a preamplification step is necessary. The first step is therefore a single-tube cDNA synthesis and preamplification reaction. Following preamplification, unincorporated primers that interfere with sequencing are removed by treatment with Applied Biosystems™ ExoSAP-IT™ PCR Product Cleanup Reagent. Next, the preamplified material is used in specific

target amplification of the S gene region. For this, the Applied Biosystems™ BigDye™ Direct Cycle Sequencing Kit and M13-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. 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.

Some of the sequences generated by this method will produce capillary electrophoresis (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.

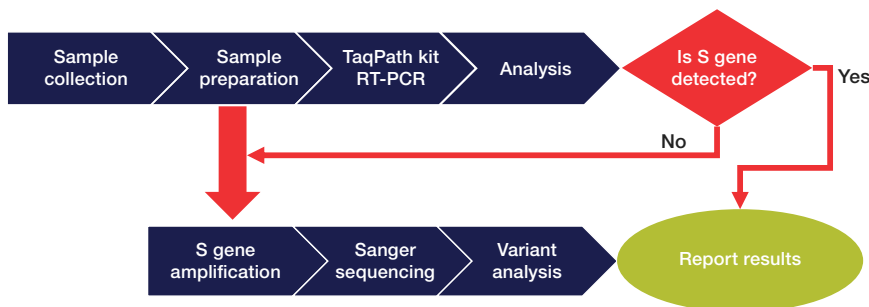


Figure 1. Workflow for Sanger sequencing detection of S gene variants. Samples are initially processed using the TaqPath COVID-19 Combo Kit. If the S gene is undetectable while other components are clearly present, the sample can be tested by Sanger sequencing for the appearance of variants in the S gene. The sample used for the TaqPath assay can be used without further processing in the Sanger sequencing protocol described here.

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

1. Materials

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 μ L to 1,000.0 μ L	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.
TaqPath 1-Step RT-qPCR Master Mix	Thermo Fisher Scientific	A15299
Nuclease-Free Water	Thermo Fisher Scientific	AM9937 or equivalent
ExoSAP-IT PCR Product Cleanup Reagent	Thermo Fisher Scientific	78200.200.UL
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

- Primers can be ordered from our custom oligo ordering web page (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 a final concentration of 100 μ M.

Sequence	Primer name
GTGTTAATCTTACAACCAGAACTCAATTAC	S_preamp_for
CACAGACTTTAATAACAACATTAGTAGCG	S_preamp_rev
TGTAAAACGACGGCCAGT CGTGGTGTATTACCCTGACAAAG	S.1.1538-M13F
CAGGAAACAGCTATGACCT TCAGTGGGAAGCAAATAAACACCCAT	S.1.1538-M13R

Note: M13 sequence tags are highlighted in red text.

1.4 Stocks of primers

- Prepare the preamplification primer mix:
 - Add 490 μ L of TE to a clean microcentrifuge tube.
 - Add 5 μ L of each of the resuspended preamplification oligonucleotides (at 100 μ M) to the tube.
 - The final tube will contain 500 μ L of solution with each primer at 1 μ M. This will be the 10X preamplification primer mix used in step 2.1.
- Prepare the target-specific amplification primer mix:
 - Add 490 μ L of TE to a clean microcentrifuge tube.
 - Add 5 μ L of oligo S.1.1538-M13F and 5 μ L of oligo S.1.1538-M13R to the tube.
 - This will be the 10X sequencing amplification primer mix, with each oligo at 1 μ M, that will be used in step 4.1.

2. cDNA synthesis and preamplification

2.1. For each sample, combine:

Reagent	Final volume		
	10 μ L	20 μ L	25 μ L
4X TaqPath 1-Step RT-qPCR Master Mix	2.5 μ L	5 μ L	6.25 μ L
10X preamplification primer mix	1 μ L	2 μ L	2.5 μ L
Sample	1–5 μ L	1–13 μ L	1–15 μ L
Water	To final 10 μ L	To final 20 μ L	To final 25 μ L

2.2. Vortex for 2 to 3 seconds, then centrifuge briefly (5 to 10 seconds) at 1,000 x *g*.

Note: Samples can be set up in 8-well tube strips or 96-well plates.

Note: Sample input volume can be adjusted for sensitivity. For example, up to 15 μ L of a sample that is expected to have low titer can be used. Reagent volumes are therefore given for three different sample input volumes.

2.3. PCR amplification

2.3.1. Program a thermal cycler with the following profile:

Parameter	Stage/step					
	Reverse transcription	Polymerase inactivation	Cycling (25 cycles)		Final extension	Hold
			Denaturation	Annealing and extension		
Temperature	50°C	95°C	95°C	60°C	72°C	4°C
Time	15 min	2 min	3 sec	45 sec	5 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. Preamplification cleanup

3.1. Remove the samples from the thermal cycler; centrifuge for 10 seconds at 1,000 x *g*.

3.2. Add 2 μ L of ExoSAP-IT reagent for each 5 μ L of preamplification volume to each tube or well:

- For 10 μ L preamplification volume, add 4 μ L ExoSAP-IT reagent
- For 20 μ L preamplification volume, add 8 μ L ExoSAP-IT reagent
- For 25 μ L preamplification volume, add 10 μ L ExoSAP-IT reagent

3.3. Label the PCR tube strip or plate “+ ExoSAP-IT”.

3.4. Seal the tubes or plate.

3.5. Vortex for 2 to 3 seconds, then centrifuge briefly (5 to 10 seconds) at 1,000 x *g*.

3.6. Place the tube strip or plate into the thermal cycler and run with the following settings:

Parameter	Stage/step		
	Nuclease digestion	Nuclease inactivation	Hold
Temperature	37°C	80°C	4°C
Time	15 min	7 min	Indefinitely

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

4. PCR amplification of targets

4.1. 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 plate setup for 22 unknown samples and two controls is shown below.

	1	2	3	4	5	6
A	Negative control S-primers	Negative control S-primers	Sample 7 S-primers	Sample 7 S-primers	Sample 15 S-primers	Sample 15 S-primers
B	Sample 1 S-primers	Sample 1 S-primers	Sample 8 S-primers	Sample 8 S-primers	Sample 16 S-primers	Sample 16 S-primers
C	Sample 2 S-primers	Sample 2 S-primers	Sample 9 S-primers	Sample 9 S-primers	Sample 17 S-primers	Sample 17 S-primers
D	Sample 3 S-primers	Sample 3 S-primers	Sample 10 S-primers	Sample 10 S-primers	Sample 18 S-primers	Sample 18 S-primers
E	Sample 4 S-primers	Sample 4 S-primers	Sample 11 S-primers	Sample 11 S-primers	Sample 19 S-primers	Sample 19 S-primers
F	Sample 5 S-primers	Sample 5 S-primers	Sample 12 S-primers	Sample 12 S-primers	Sample 20 S-primers	Sample 20 S-primers
G	Sample 6 S-primers	Sample 6 S-primers	Sample 13 S-primers	Sample 13 S-primers	Sample 21 S-primers	Sample 21 S-primers
H	Positive control S-primers	Positive control S-primers	Sample 14 S-primers	Sample 14 S-primers	Sample 22 S-primers	Sample 22 S-primers

4.2. In a 96-well PCR plate, combine:

- 1 μ L of 10X M13-tailed target-specific amplification primer mix (see section 1.4) in duplicate (“S-primers”, as suggested in table above)
- 5 μ L of 2X BigDye Direct PCR Master Mix (supplied in kit)
- 1–3 μ L of cDNA sample treated with ExoSAP-IT reagent, from step 3.6
 - Leftover cDNA sample can be frozen at -20°C .
- Water to 10 μ L total volume

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

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

Parameter	Stage/step				
	Polymerase activation	Cycling (35 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 μ L total reaction volume. The cycle sequencing and BigDye XTerminator steps have been optimized for 10 μ L input volumes.

5. Cycle sequencing

5.1. Once the PCR in step 4.4 is complete, the plate can be used directly for cycle sequencing.

5.2. Remove the seal from the plate.

5.3. Add to each well:

- 2 μ L of BigDye Direct Sequencing Master Mix (supplied in kit)
- 1 μ L 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 previous plate setup is shown below.

	1	2	3	4	5	6
A	Negative control M13-For	Negative control M13-Rev	Sample 7 M13-For	Sample 7 M13-Rev	Sample 15 M13-For	Sample 15 M13-Rev
B	Sample 1 M13-For	Sample 1 M13-Rev	Sample 8 M13-For	Sample 8 M13-Rev	Sample 16 M13-For	Sample 16 M13-Rev
C	Sample 2 M13-For	Sample 2 M13-Rev	Sample 9 M13-For	Sample 9 M13-Rev	Sample 17 M13-For	Sample 17 M13-Rev
D	Sample 3 M13-For	Sample 3 M13-Rev	Sample 10 M13-For	Sample 10 M13-Rev	Sample 18 M13-For	Sample 18 M13-Rev
E	Sample 4 M13-For	Sample 4 M13-Rev	Sample 11 M13-For	Sample 11 M13-Rev	Sample 19 M13-For	Sample 19 M13-Rev
F	Sample 5 M13-For	Sample 5 M13-Rev	Sample 12 M13-For	Sample 12 M13-Rev	Sample 20 M13-For	Sample 20 M13-Rev
G	Sample 6 M13-For	Sample 6 M13-Rev	Sample 13 M13-For	Sample 13 M13-Rev	Sample 21 M13-For	Sample 21 M13-Rev
H	Positive control M13-For	Positive control M13-Rev	Sample 14 M13-For	Sample 14 M13-Rev	Sample 22 M13-For	Sample 22 M13-Rev

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

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

Parameter	Stage/step						
	Post-PCR cleanup	Post-PCR inactivation	Polymerase activation	Cycling (25 cycles)			Hold
				Denaturation	Annealing	Extension	
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

6. Sequencing cleanup

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

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

6.2.1. 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.

6.2.2. 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.

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

6.2.4. 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.

6.2.5. Mix the tube of combined reagents until homogeneous.

6.3. 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.

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

6.5. Vortex the reaction plate for 40 minutes.

6.6. In a swinging-bucket centrifuge, spin the plate at 1,500 x *g* for 2 minutes.

7. Collect data

7.1. 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.

7.2. Remove the MicroAmp Optical Adhesive Film and replace the film with a 96-well plate septum.

7.3. Load plates into the genetic analyzer.

7.4. 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_POP7xI_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
 - ShortSeqBDX

8. Analyze results using a sequencing program

Sequence Scanner 2 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.

8.1. To obtain the software, go to <http://resource.thermofisher.com/pages/WE28396/>

8.2. Using Sequence Scanner 2 software, generate a QC report. For each sequencing trace, determine the trace score, CRL, and the QV20+ score.

8.3. 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 previous criteria are indeterminate and should be repeated.

8.4. 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.

8.5. For variant analysis of the S gene, these criteria should be met:

- Positive (passable) traces in both directions (section 8.3)
- Homology to the SARS-CoV-2 genome (section 8.4) in regions outside putative variant
- Negative traces in no-template control reactions (section 8.3)

8.6. 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).

References

1. Wise J (2020) Covid-19: New coronavirus variant is identified in UK. *BMJ* Dec 16;371:m4857. doi: 10.1136/bmj.m4857
2. Public Health England (2020) Investigation of novel SARS-COV-2 variant: Variant of concern 202012/01. Technical briefing 1. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/947048/Technical_Briefing_VOC_SH_NJL2_SH2.pdf
3. Rambaut A et al. (2020) Preliminary genomic characterisation of an emergent SARS-CoV-2 lineage in the UK defined by a novel set of spike mutations. <https://virological.org/t/preliminary-genomic-characterisation-of-an-emergent-sars-cov-2-lineage-in-the-uk-defined-by-a-novel-set-of-spike-mutations/563>

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