Protocol for Sanger sequencing of any region of the SARS-CoV-2 genome

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 mutates quickly, 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 also may 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]. New lineages continue to appear that are predicted to have increased infectivity, resistance to treatments, or both [2,3]. In fact, the B.1.1.7 strain was recently shown to have increased mortality relative to the wild-type strain [4]. It is therefore critically important for global surveillance efforts to understand what, how, and when new mutations arise.

Sequence-based surveillance methods provide the simplest and most direct paths to identifying and tracing strain lineages. Thermo Fisher Scientific has developed methods for strain identification on its Ion Torrent™ NGS platform, Applied Biosystems™ TaqMan® RT-qPCR platform, and Applied Biosystems™ capillary electrophoresis (CE)-based genetic analyzer platform. Each of these strategies provides slightly different advantages. In general, NGS-based approaches are ideal for unbiased **discovery** of new mutations in the whole genome, the CE-based approach is ideal for **focused** analysis of specific regions of the genome, and RT-qPCR approaches are ideal for highly specific **detection** of well-defined sequences and mutations. Thus, these three approaches have complementary strengths and each fulfills a need for carrying out surveillance studies. For more information, see **the behind-the-bench blog on SARS-CoV-2 surveillance** or talk to your local Thermo Fisher Scientific representative.

To provide methods for CE-based focus on strain lineages, we developed Sanger sequencing protocols for specific amplicons that can be used to confirm mutations found in some strain lineages. However, a protocol that facilitates flexible choices for confirming any mutation is needed. We therefore developed tools for selecting primer sequences for any position in the SARS-CoV-2 genome, and a protocol for using them for variant analysis via Sanger sequencing. Briefly, cDNA is synthesized from a sample containing viral RNA. Next, the cDNA is used in target amplification of specific regions using M13 sequence–tagged primers that cover the regions of interest. 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 M13-forward and 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 generated by standard CE. The sequences obtained can be read by any sequencing program, such as Applied Biosystems™ SeqA software (Figure 1).

Further comparison to the SARS-CoV-2 reference genomic sequence that can be used to identify and confirm variants is possible with Applied Biosystems™ SeqScape™ or Applied Biosystems™ Variant Reporter™ Software [5].

Figure 1. Workflow to detect 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 for SARS-CoV-2 identity and compared to reference sequences to determine if variants are present.

Note: This protocol and the reagents described within are 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
One of the following:		
Digital Vortex Mixer	Thermo Fisher Scientific	88882010
IKA MS 3 Digital Shaker	Thermo Fisher Scientific	QP0706
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	Any major laboratory supplier	
Cold block or ice		
Plate centrifuge		
Microcentrifuge or mini centrifuge		
Vortex mixer		

1.2 Reagents, kits, and consumables

Product	Supplier	Cat. No.
SuperScript IV VILO Master Mix	Thermo Fisher Scientific	117565500
Nuclease-free water		AM9937 or equivalent
BigDye Direct Cycle Sequencing Kit		4458688 or equivalent
BigDye XTerminator Purification Kit		4376486 or equivalent
MicroAmp Fast Optical 96-Well Reaction Plate with Barcode		4346906 or 4366932
MicroAmp Optical Adhesive Film		4306311
Nonstick, RNase-free microcentrifuge tubes, 1.5 mL		AM12450 or equivalent
5mL tube, PCR clean	Any major laboratory supplier	
Sterilized aerosol barrier (filter) pipette tips		

1.3. Primers

- Choose primers from either the SARS-CoV-2 Sanger Primer Visualization App or the SARS-CoV-2 Sanger Primer Pair Lookup App (see Appendix).
- Once sequences are defined, 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 of 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 steps 3.1–3.2.

2. cDNA synthesis

2.1. RNA purified from samples (using methods of any major purification kit) should be resuspended in nuclease-free water.

2.2. 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

Note: Sample input volume can be adjusted for sensitivity. For example, if a sample is expected to have low titer, up to 15 µL may be used.

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

2.4. Reverse transcription

2.4.1. Program a thermal cycler with the following profile:

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

2.4.2. 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 amplification 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 six samples and six primer pairs is shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	SC2M1-55 primers	SC2M1-55 primers	SC2M1-55 primers	SC2M1-55 primers	SC2M1-55 primers	SC2M1-55 primers	SC2M1-55 primers	SC2M1-55 primers	SC2M1-55 primers	SC2M1-55 primers	SC2M1-55 primers	SC2M1-55 primers
B	SC2M1-58 primers	SC2M1-58 primers	SC2M1-58 primers	SC2M1-58 primers	SC2M1-58 primers	SC2M1-58 primers	SC2M1-58 primers	SC2M1-58 primers	SC2M1-58 primers	SC2M1-58 primers	SC2M1-58 primers	SC2M1-58 primers
C	SC2M1-59 primers	SC2M1-59 primers	SC2M1-59 primers	SC2M1-59 primers	SC2M1-59 primers	SC2M1-59 primers	SC2M1-59 primers	SC2M1-59 primers	SC2M1-59 primers	SC2M1-59 primers	SC2M1-59 primers	SC2M1-59 primers
D	SC2M1-60 primers	SC2M1-60 primers	SC2M1-60 primers	SC2M1-60 primers	SC2M1-60 primers	SC2M1-60 primers	SC2M1-60 primers	SC2M1-60 primers	SC2M1-60 primers	SC2M1-60 primers	SC2M1-60 primers	SC2M1-60 primers
E	SC2M1-62b primers	SC2M1-62b primers	SC2M1-62b primers	SC2M1-62b primers	SC2M1-62b primers	SC2M1-62b primers	SC2M1-62b primers	SC2M1-62b primers	SC2M1-62b primers	SC2M1-62b primers	SC2M1-62b primers	SC2M1-62b primers
F	SC2M1-64 primers	SC2M1-64 primers	SC2M1-64 primers	SC2M1-64 primers	SC2M1-64 primers	SC2M1-64 primers	SC2M1-64 primers	SC2M1-64 primers	SC2M1-64 primers	SC2M1-64 primers	SC2M1-64 primers	SC2M1-64 primers

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

Note: The layout above is an example based on six primer pairs that query mutations found in B.1.1.7_E484K S-gene across six samples. Modify the layout as needed, adjusting for the primer pairs that will be used and the number of samples that will be analyzed.

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 µL of 10X sequencing amplification primer mix in duplicate (as suggested in the table in step 3.1)
- 5 µL of 2X BigDye Direct PCR Master Mix (supplied in kit)
- 1 µL of cDNA sample from completed step 2.4
  - 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:

	Stage/step				
		Cycling (40 cycles)			
Parameter	Polymerase activation	Denaturation	Annealing	Extension	Hold
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 reaction setup.

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 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 shown in step 3.1 above is shown below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	SC2M1-55 M13 For	SC2M1-55 M13 Rev	SC2M1-55 M13 For	SC2M1-55 M13 Rev	SC2M1-55 M13 For	SC2M1-55 M13 Rev	SC2M1-55 M13 For	SC2M1-55 M13 Rev	SC2M1-55 M13 For	SC2M1-55 M13 Rev	SC2M1-55 M13 For	SC2M1-55 M13 Rev
B	SC2M1-58 M13 For	SC2M1-58 M13 Rev	SC2M1-58 M13 For	SC2M1-58 M13 Rev	SC2M1-58 M13 For	SC2M1-58 M13 Rev	SC2M1-58 M13 For	SC2M1-58 M13 Rev	SC2M1-58 M13 For	SC2M1-58 M13 Rev	SC2M1-58 M13 For	SC2M1-58 M13 Rev
C	SC2M1-59 M13 For	SC2M1-59 M13 Rev	SC2M1-59 M13 For	SC2M1-59 M13 Rev	SC2M1-59 M13 For	SC2M1-59 M13 Rev	SC2M1-59 M13 For	SC2M1-59 M13 Rev	SC2M1-59 M13 For	SC2M1-59 M13 Rev	SC2M1-59 M13 For	SC2M1-59 M13 Rev
D	SC2M1-60 M13 For	SC2M1-60 M13 Rev	SC2M1-60 M13 For	SC2M1-60 M13 Rev	SC2M1-60 M13 For	SC2M1-60 M13 Rev	SC2M1-60 M13 For	SC2M1-60 M13 Rev	SC2M1-60 M13 For	SC2M1-60 M13 Rev	SC2M1-60 M13 For	SC2M1-60 M13 Rev
E	SC2M1-62b M13 For	SC2M1-62b M13 Rev	SC2M1-62b M13 For	SC2M1-62b M13 Rev	SC2M1-62b M13 For	SC2M1-62b M13 Rev	SC2M1-62b M13 For	SC2M1-62b M13 Rev	SC2M1-62b M13 For	SC2M1-62b M13 Rev	SC2M1-62b M13 For	SC2M1-62b M13 Rev
F	SC2M1-64 M13 For	SC2M1-64 M13 Rev	SC2M1-64 M13 For	SC2M1-64 M13 Rev	SC2M1-64 M13 For	SC2M1-64 M13 Rev	SC2M1-64 M13 For	SC2M1-64 M13 Rev	SC2M1-64 M13 For	SC2M1-64 M13 Rev	SC2M1-64 M13 For	SC2M1-64 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 (40 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 beads needed for all samples. You will need 45 µL of SAM Solution and 10 µL of BigDye XTerminator Solution per well, plus an extra 10% to account for pipetting accuracy. For example, for a 96-well plate, you will need 4,752 µL of SAM Solution and 1,056 µL of BigDye XTerminator Solution in total.

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 BigDye 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/BigDye XTerminator Solution mix every 8–10 wells to homogenize the bead mixture.

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

5.5. Vortex the reaction plate for 40 minutes. If using the Thermo Scientific™ Digital Vortex Mixer, vortex at 1,800 rpm; for the TAITEC vortexer, use the maximum speed.

5.6. In a swinging-bucket centrifuge, spin the plate at 1,500 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/or 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 Clear Adhesive 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 sequence data quality and variant identification

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

Note: Other software packages that can be used to analyze the primary sequencing traces are Applied Biosystems™ Sequencing Analysis Software v6.0 and the Thermo Fisher™ Connect Platform–based Sanger Quality Check app. Refer to the documentation for those packages for specific instructions.

7.1. To obtain the Sequence Scanner v2.0 software, go to:
http://resource.thermofisher.com/page/WE28396_2/

7.2. Using Sequence Scanner v2.0 software, generate a QC report. For each sequencing trace, determine the trace score, contiguous read length (CRL), and number of bases with basecaller quality value greater than or equal to 20 (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™ or another sequence alignment tool, align positive traces to the SARS-CoV-2 genome.

- Alignments greater than 85% identity 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 validity, failures, and retests

- Test runs that fail for reasons not attributable to the system performance, such as equipment malfunction, operator error, or other demonstrable cause, should be considered invalid runs. Invalid runs should be retested and documented in any relevant study reports.

7.7. Variant analysis

- Two Applied Biosystems™ software solutions for variant analysis are available.
  - SeqScape 4.0 Software is a sequencing package designed for mutation detection and analysis, single-nucleotide polymorphism (SNP) discovery and validation, pathogen subtyping, allele identification, and sequence confirmation. It provides library functions for comparison to a known group of sequences, as well as 21 CFR Part 11 functionalities. A demo version of the software can be accessed from **https://www.thermofisher.com/order/catalog/product/A38880?us&en#/A38880?us&en**. A quick-start guide for using SeqScape 4.0 Software, including brief instructions, reference sequence, and analysis files, can be downloaded from **https://www.thermofisher.com/us/en/home/life-science/sequencing/sanger-sequencing/applications/sars-cov-2-research.html**.

– Variant Reporter 3.0 Software is designed for reference-based and non–reference-based sequence comparisons, such as mutation detection and analysis, SNP discovery and validation, and sequence confirmation. An initial license for Variant Reporter 3.0 Software can be requested at **https://www.thermofisher.com/order/catalog/product/A38884?us&en#/A38884?us&en**. A quick-start guide for using Variant Reporter Software, including brief instructions, reference sequence, and analysis files, can be downloaded from **https://www.thermofisher.com/us/en/home/life-science/sequencing/sanger-sequencing/applications/sars-cov-2-research.html**.

For detailed instruction for using either of these programs, see the appropriate User Guide.

Appendix—tools for choosing Sanger sequencing primers in SARS-CoV-2 genome analysis

Choosing Sanger sequencing primers for specific strain lineages or regions of SARS-CoV-2 can be complicated. Thermo Fisher Scientific has developed two tools to assist in the selection of primers that can be used to analyze any region in the SARS-CoV-2 genome by Sanger sequencing. One of these is the SARS-CoV-2 Sanger Primer Visualization App based on the open-source web-app platform JBrowse 2 [6]. This visualization tool can be used to view sequences in the context of the genome and choose primer sets that can be used to sequence those regions (Figures 2–7).

The second is a text-based lookup application that accepts strain type, mutation, or genome coordinate inputs and identifies the appropriate primer sets for those inputs (Figures 8–12). Both tools contain the set of primer sequences referenced by the Centers for Disease Control and Prevention [7] or optimized by Thermo Fisher Scientific, preloaded into their databases. All the primers have been functionally tested. In some cases, primers for a region needed a redesign in order to pass the functional tests.

Using the SARS-CoV-2 Sanger Primer Visualization App

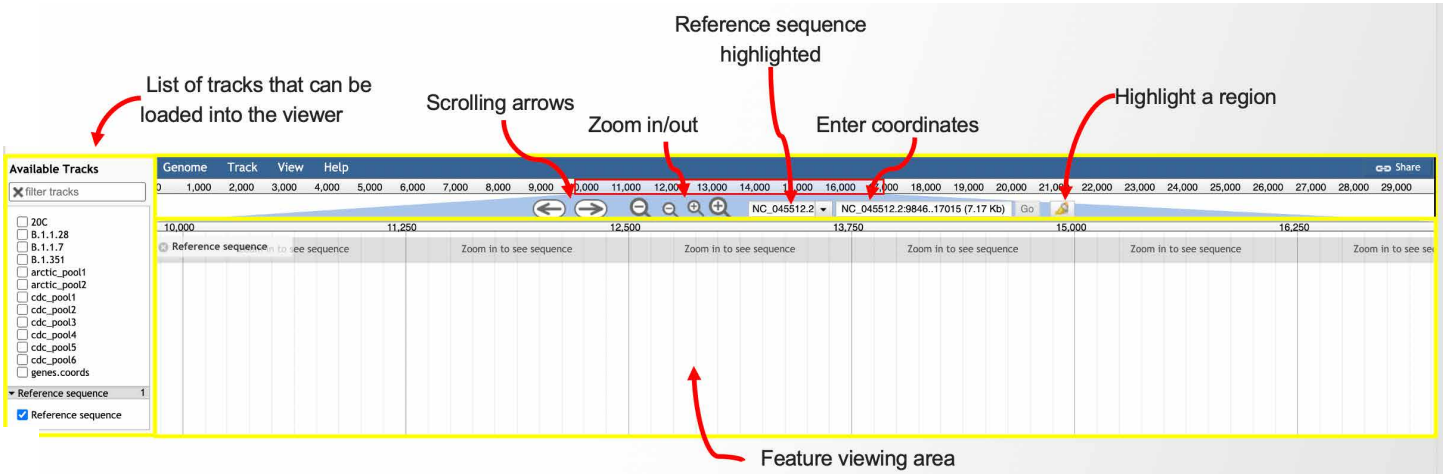


Figure 2. Navigating the visual browser. The browser can be accessed at **https://www.thermofisher.com/us/en/home/life-science/sequencing/sanger-sequencing/applications/sars-cov-2-research.html**. When browser loading is complete, a screen similar to the one pictured above will appear. The browser is broken into three major sections (yellow boxes). The top center of the browser is used for navigation. Here, the scrolling arrows can be used to change the viewable area and the magnifying glasses can be used to zoom in or out on a region. The reference sequence is shown in the center field and the field to the right of that can be used to enter and zoom to specific genomic coordinates. On the far left side of the screen, a window shows the available tracks that can be visualized. Clicking in the boxes will add tracks to the feature viewing area. The reference sequence for the SARS-CoV-2 wild type strain (NC_045512.2) is preloaded into the browser.

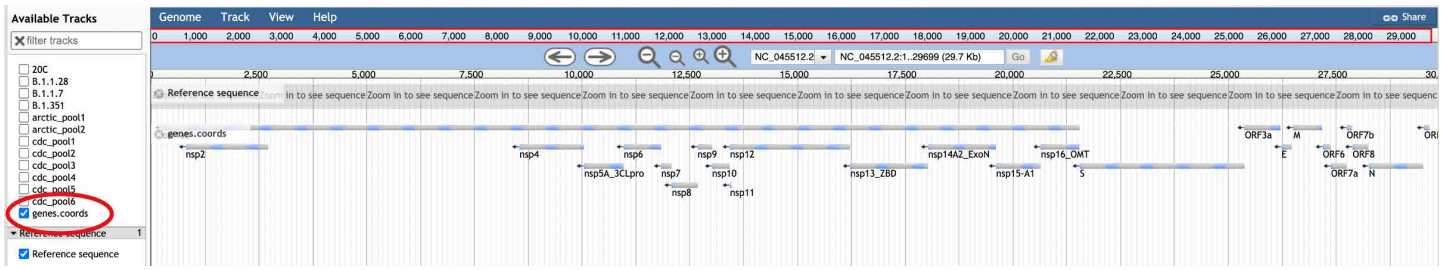


Figure 3. Preloading of genomic coordinates for all of the SARS-CoV-2 genes into a track (genes.coords). Clicking on a box in the Available Tracks window (red circle) will add the track to the viewing area. Note that each gene is depicted as a gray and blue bar, with the direction of transcription shown with a small arrow.

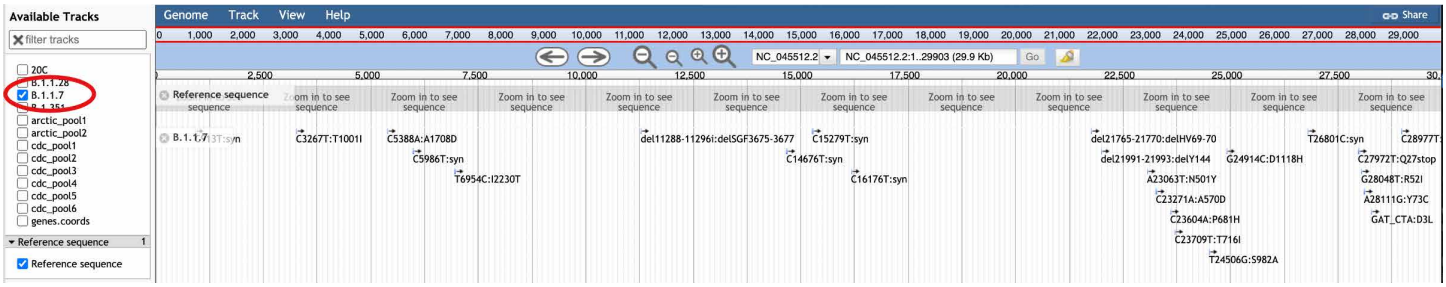


Figure 4. Preloading of tracks for variants of concern (VOCs) into the browser. Clicking on a box in the Available Tracks window (red circle) will add the mutations to the viewing area. Each mutation is shown with the changes in both the nucleotide and amino acid sequences. In the example shown above, the mutations defining the B.1.1.7 strain lineage are shown. Note that multiple boxes can be chosen, and multiple tracks can be viewed in the viewing area. The database of VOCs preloaded will be updated biweekly.

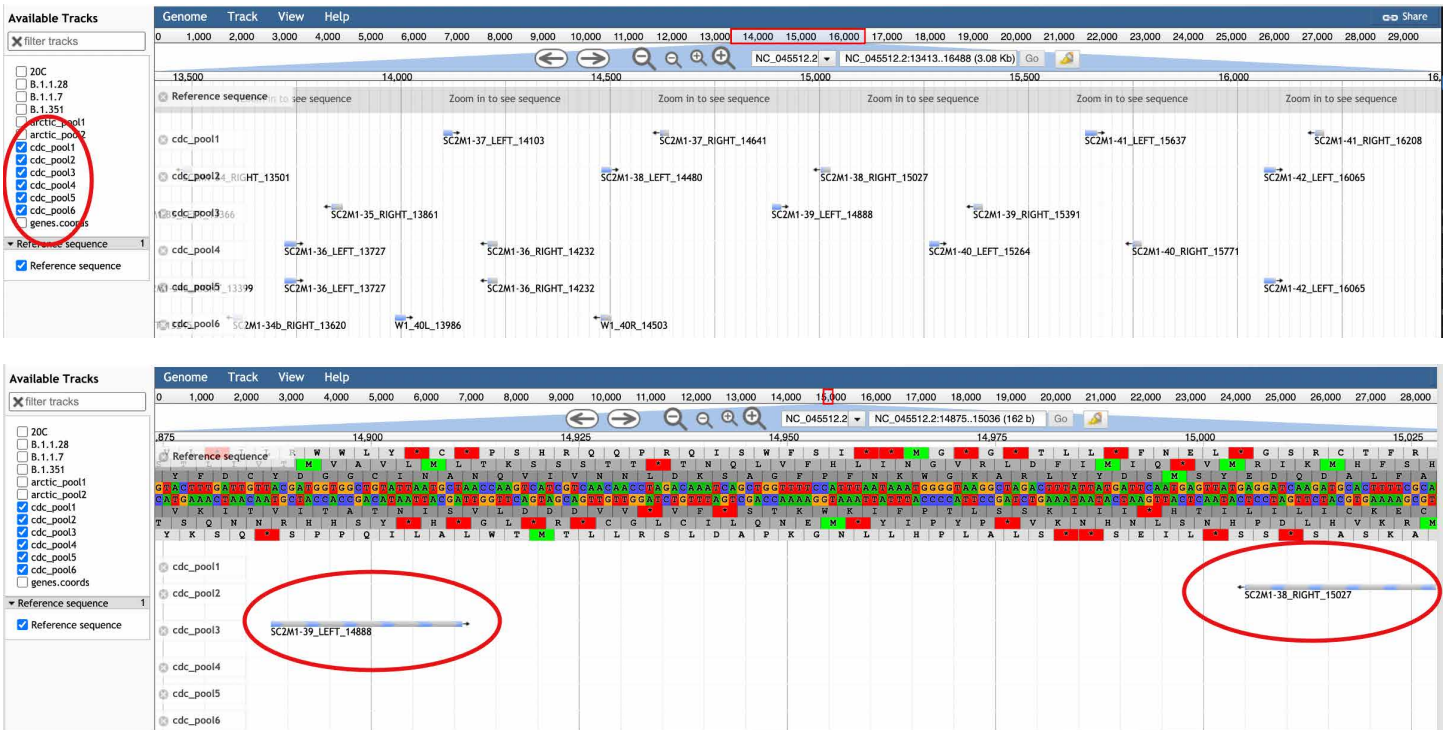


Figure 5. Primers listed in the publication by the CDC [4], redesigned by Thermo Fisher Scientific based on results from functional testing, and preloaded into the browser. Clicking on boxes in the Available Tracks window will display the primers in the viewing area. Primers are shown as gray and blue bars, with arrows indicating the 5′ to 3′ direction. A pair of sequencing primers is designated by a prefix SC2M1, followed by a number for the pair, whether the primer is left or right, and the coordinates of the nucleotide that is closest to the 5′ end. The top figure shows a zoomed-out view of the region with primers falling between the 13413–16488 coordinates. In the bottom figure, a zoomed-in view is shown, highlighting two primer pairs (red circles, SC2M1-39_LEFT_14888 and SC2M1-38_RIGHT_15207).

Figure 6. Finding primer sets. To find primers for a specific mutation, select the variant(s) of interest and primer pool tracks in the Available Tracks window. For clarity, a zoomed-in view of the region around the C14676T:syn mutation (circled in blue) is shown in the viewing area. Note that there are two primers that can generate an amplicon encompassing this mutation: SC2M1-38_LEFT_14480 and SC2M1-38_RIGHT_15027 (circled in red). These can be ordered and used to sequence the region encompassing this mutation. Note that there are other primers that fall within this view (SC2M1-37_RIGHT_14641) but that are not useful for generating an amplicon that covers the mutation of interest. Alternatively, primers for specific coordinates (i.e., not defined by a mutation) can be found either by using the navigation arrows to scroll to the region, or by entering the coordinates of the nucleotide into the coordinate field.

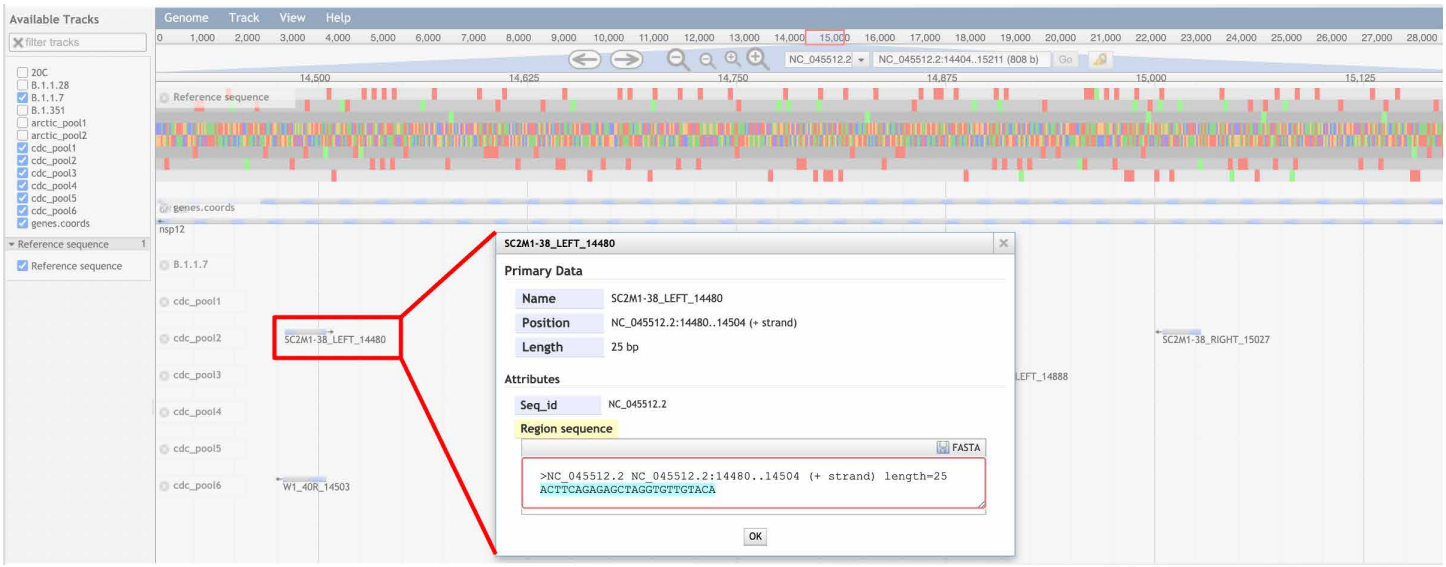


Figure 7. Determining the primer sequence. The sequence of a particular feature can be seen by clicking on that feature's blue/gray box or name in the viewing area. The cursor will change to a pointer when hovered over a clickable feature; upon clicking, a new window with details about that feature will appear. Here, clicking on SC2M1-38_LEFT_14480 brought up the window shown. The sequence of the primer is shown highlighted in blue. This text can be cut and pasted into an ordering webpage or form, or downloaded as a FASTA file. Note that to use the Sanger sequencing protocol, M13 sequences must be appended to the 5′ end of each primer. For example, to use the SC2M1 set, primers must be ordered as **TGTAAAACGACGGCCAGT**ACTTCAGAGAGCTAGGTGTTGTACA (left) and **CAGGAAACAGCTATGACC**TGCGAAAAGTGCATCTTGATCCT (right). The M13 sequences (**TGTAAAACGACGGCCAGT** (forward) and **CAGGAAACAGCTATGACC** (reverse)) in the primers are highlighted with bold text.

Using the SARS-CoV-2 Sanger Primer Pair Lookup App

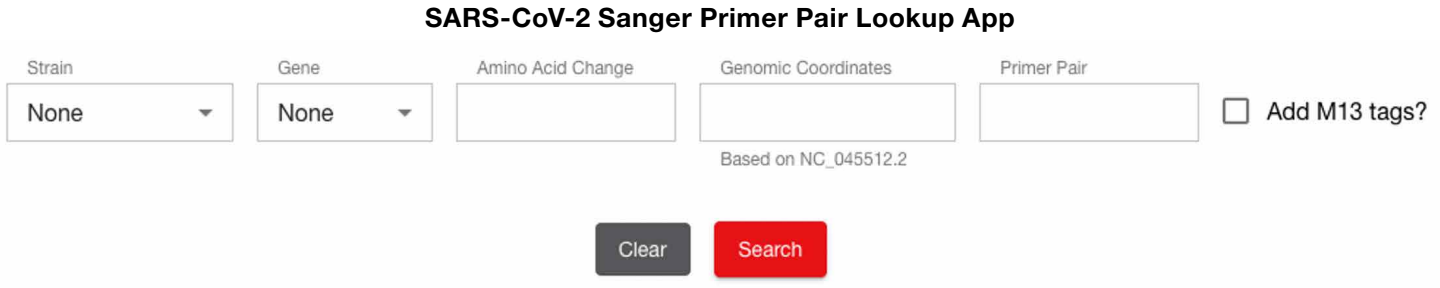


Figure 8. Primers found by using the lookup app. This tool can be accessed by going to **https://www.thermofisher.com/us/en/home/life-science/sequencing/sanger-sequencing/applications/sars-cov-2-research.html**. Functionally tested primers that cover the desired feature(s) are preloaded into the app. These include strain lineages, SARS-CoV-2 genes, mutations that produce amino acid changes, and genomic coordinates. The sequences of preloaded primer pairs can also be retrieved from the app. An option for appending M13 sequences to the primers is also provided by clicking the check box.

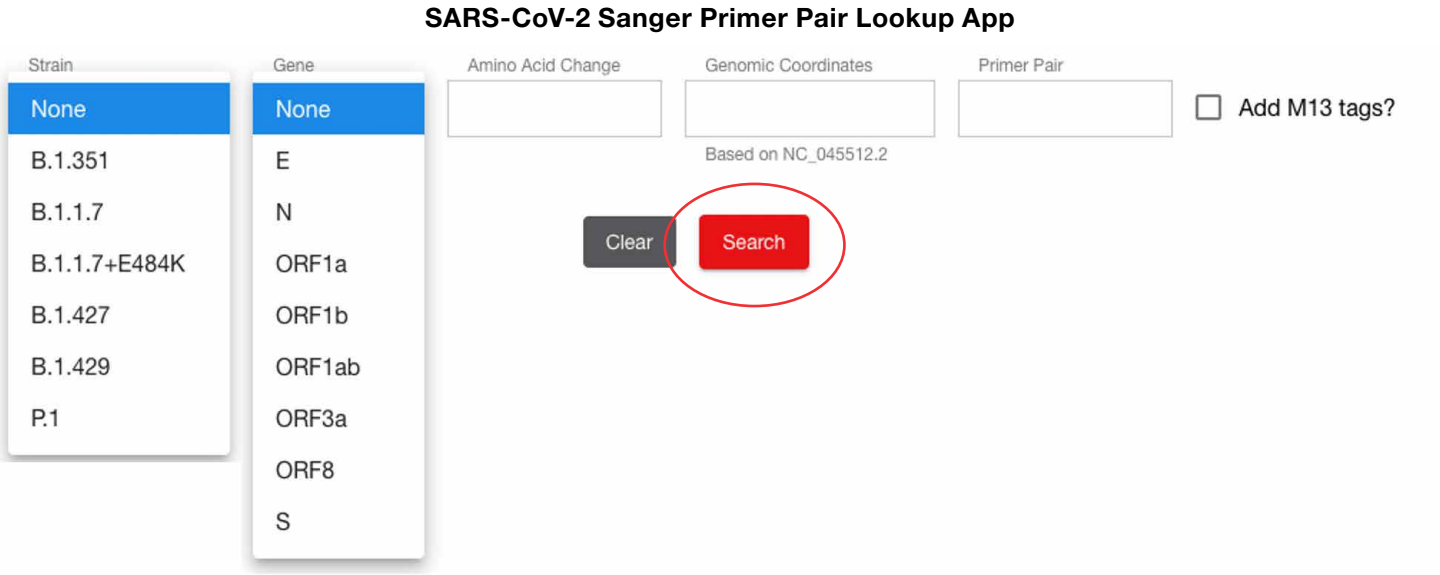
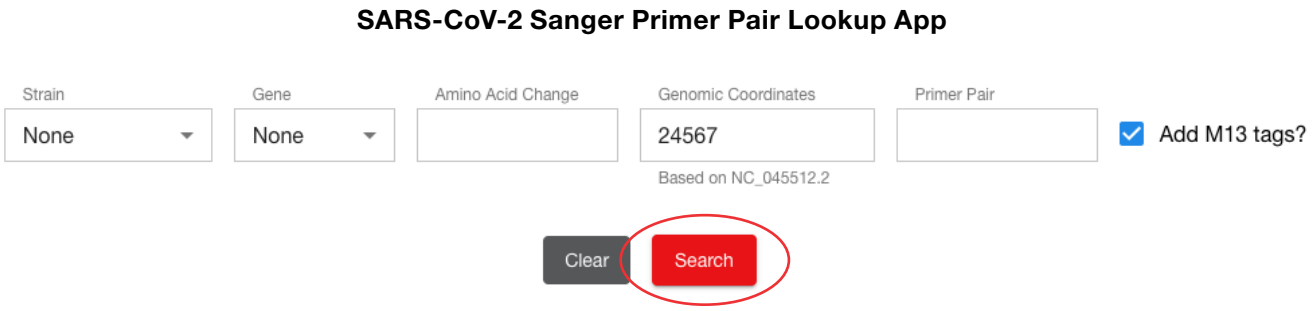


Figure 9. Using the SARS-CoV-2 Primer Pair Lookup App to preload primer sequences for strain lineages and genes. These are accessed using the drop-down menu that appears when clicking in the box. Note that both drop-down menus are shown in this figure for illustrative purposes, but in actual use only a single drop-down menu will appear at a time. Once a choice is made, click Search. The strain lineages preloaded into the app's database are VOCs and will be updated biweekly to account for new lineages as they arise.

A



B

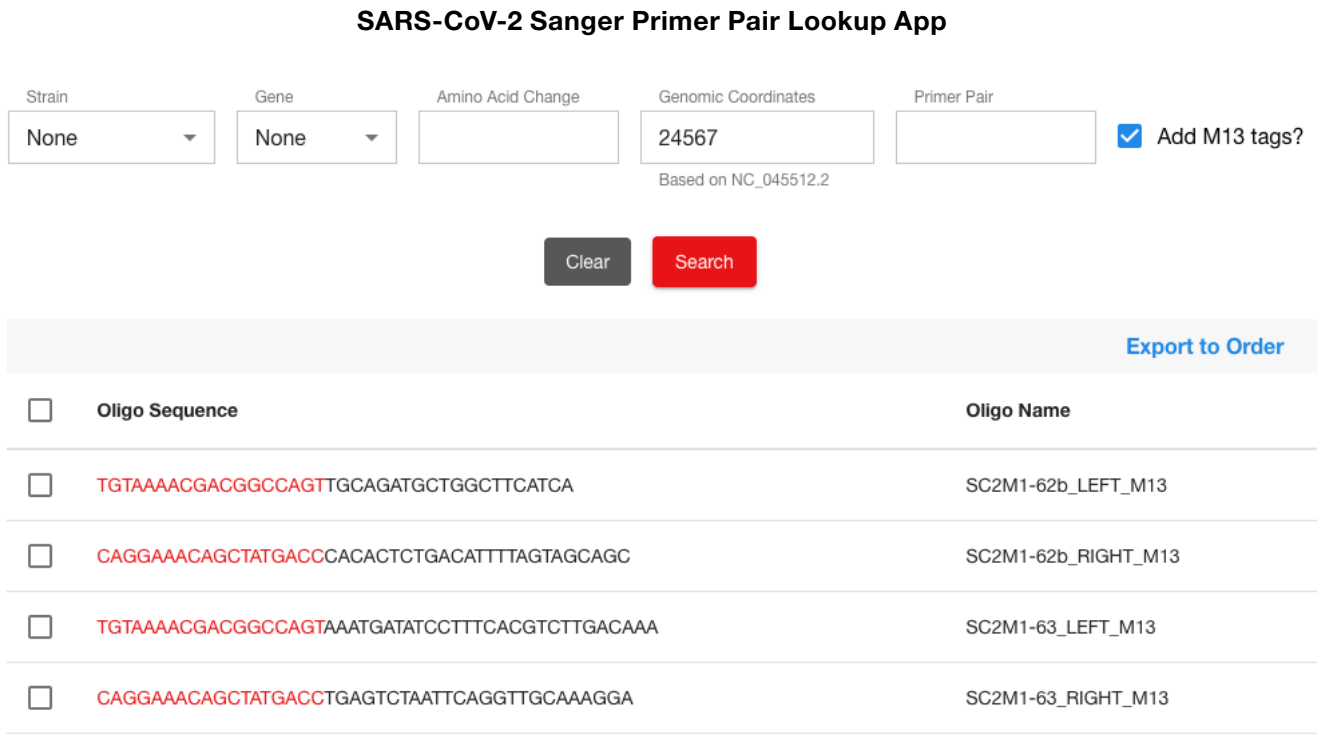
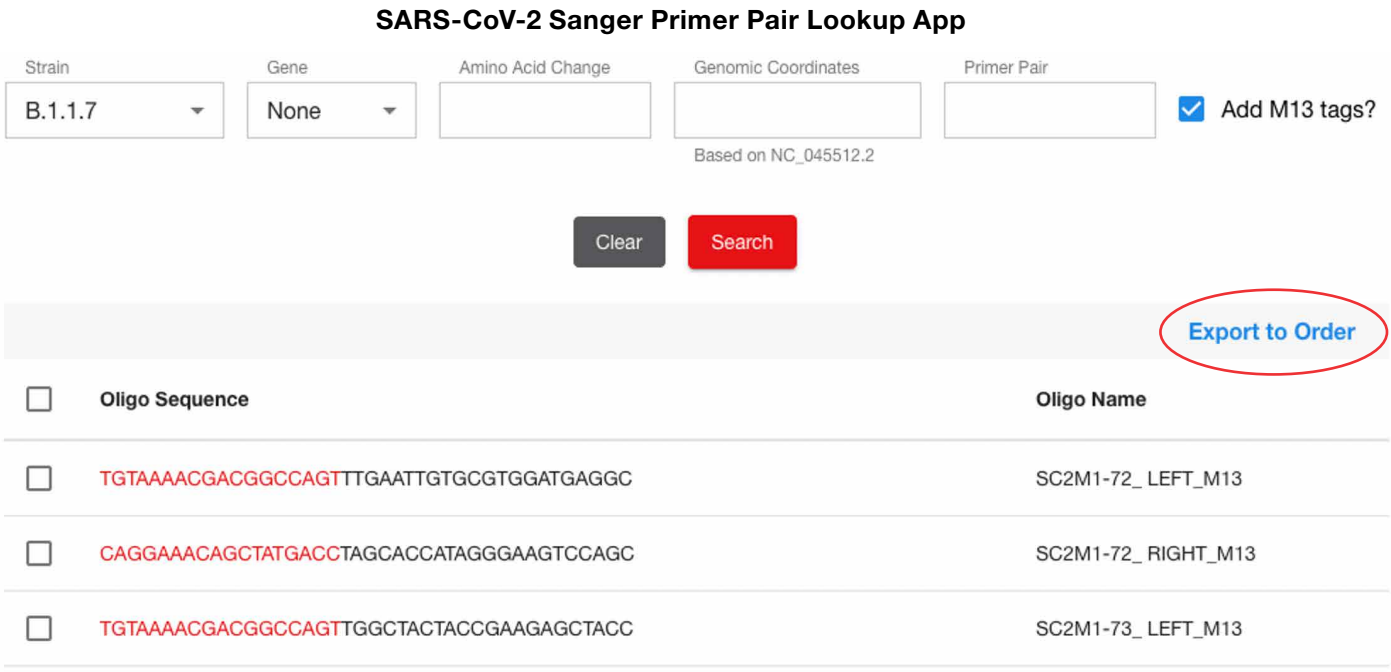
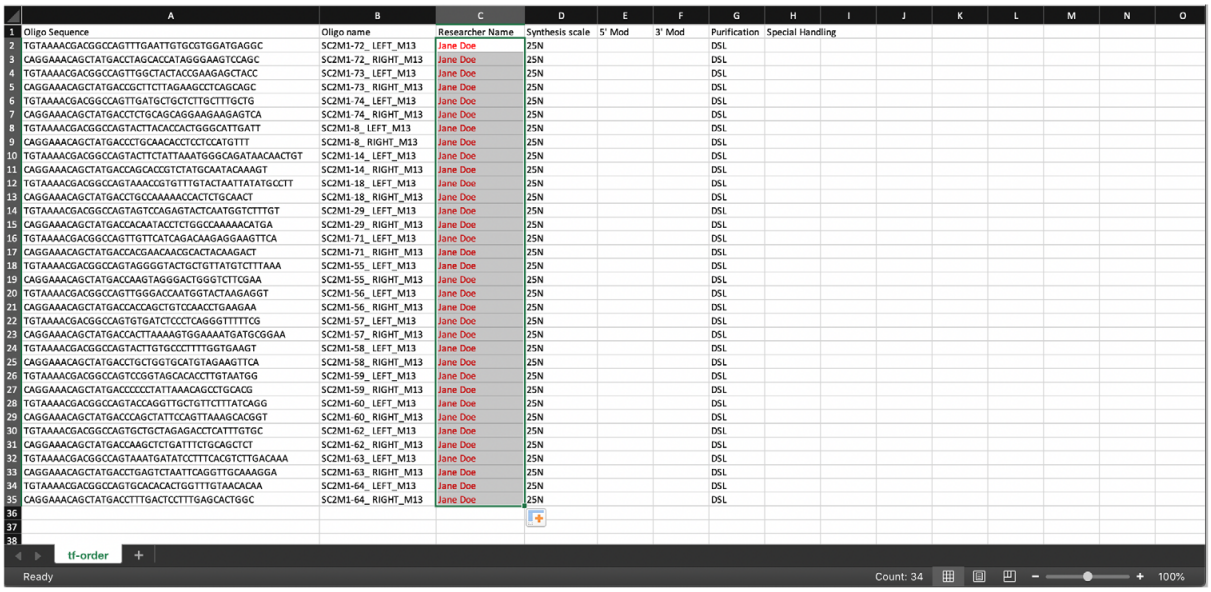


Figure 10. Entering text in the Amino Acid Change, Genomic Coordinates, and Primer Pair fields. In the example shown, **(A)** entering 24567 followed by checking the box for "Add M13 tags?" and clicking Search returns **(B)** two sets of primers, SC2M1-62 and SC2M1-63. Either of these could be used to sequence across the coordinate entered. The M13 sequences are shown in red text; the SARS-CoV-2 sequences are in black text. Note that when entering text in the Amino Acid Change field, it should be in the form of XyyyZ—for example, N501Y for an asparagine to tyrosine change at position 501. Since amino acid changes only associated with strain lineages are entered, specifying the gene affected is not necessary. For novel mutations resulting in amino acid changes, use the genomic coordinate of the corresponding nucleotide change to search for primers.

A



B



Oligo Sequence	Oligo name	Researcher Name	Synthesis scale	5' Mod	3' Mod	Purification	Special Handling
TGTAAAACGACGGCCAGTTTGAATTGTGCGTGGATGAGGC	SC2M1-72_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCTAGCACCATAGGGAAGTCCAGC	SC2M1-72_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTTGGCTACTACCGAAGAGCTACC	SC2M1-73_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCGCTTCTTAGAAGCCTCAGCAGC	SC2M1-73_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTTGATGCTGCTCTTGCTTTGCTG	SC2M1-74_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCTCTGCAGCAGGAAGAAGAGTCA	SC2M1-74_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTACTTACACCACTGGGCATTGATT	SC2M1-8_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCCTGCAACACCTCCTCCATGTTT	SC2M1-8_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTACTTCTATTAAATGGGCAGATAACAACTGT	SC2M1-14_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCAGCACCGTCTATGCAATACAAAGT	SC2M1-14_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTAAACCGTGTTTGTACTAATTATATGCCTT	SC2M1-18_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCTGCCAAAAACCACTCTGCAACT	SC2M1-18_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTAGTCCAGAGTACTCAATGGTCTTTGT	SC2M1-29_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCACAATACCTCTGGCCAAAAACATGA	SC2M1-29_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTTGTTCATCAGACAAGAGGAAGTTCA	SC2M1-71_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCACGAACAACGCACTACAAGACT	SC2M1-71_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTAGGGGTACTGCTGTTATGTCTTTAAA	SC2M1-55_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCAAGTAGGGACTGGGTCTTCGAA	SC2M1-55_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTTGGGACCAATGGTACTAAGAGGT	SC2M1-56_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCACCAGCTGTCCAACCTGAAGAA	SC2M1-56_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTGTGATCTCCCTCAGGGTTTTTCG	SC2M1-57_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCACTTAAAAGTGGAAAATGATGCGGAA	SC2M1-57_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTACTTGTGCCCTTTTGGTGAAGT	SC2M1-58_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCTGCTGGTGCATGTAGAAGTTCA	SC2M1-58_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTCCGGTAGCACACCTTGTAATGG	SC2M1-59_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCCCCCTATTAAACAGCCTGCACG	SC2M1-59_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTACCAGGTTGCTGTTCTTTATCAGG	SC2M1-60_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCCAGCTATTCCAGTTAAAGCACGGT	SC2M1-60_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTGCTGCTAGAGACCTCATTTGTGC	SC2M1-62_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCAAGCTCTGATTTCTGCAGCTCT	SC2M1-62_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTAAATGATATCCTTTCACGTCTTGACAAA	SC2M1-63_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCTGAGTCTAATTCAGGTTGCAAAGGA	SC2M1-63_ RIGHT_M13	Jane Doe	25N			DSL	
TGTAAAACGACGGCCAGTGCACACACTGGTTTGTAACACAA	SC2M1-64_ LEFT_M13	Jane Doe	25N			DSL	
CAGGAAACAGCTATGACCTTTGACTCCTTTGAGCACTGGC	SC2M1-64_ RIGHT_M13	Jane Doe	25N			DSL	

Figure 11. Primer list can be exported for ordering. (A) Clicking on Export to Order will generate a .csv text file. This can be opened in Microsoft™ Excel™ format **(B)**, modified as needed, and uploaded to a web page ordering form. The default synthesis scale and purification type, which are 25 nmol and desalted (DSL), respectively, are preloaded. An entry must be added to the Researcher Name field before uploading to the Thermo Fisher Scientific oligo ordering site (**https://www.thermofisher.com/order/custom-standard-oligo**).

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biosystems

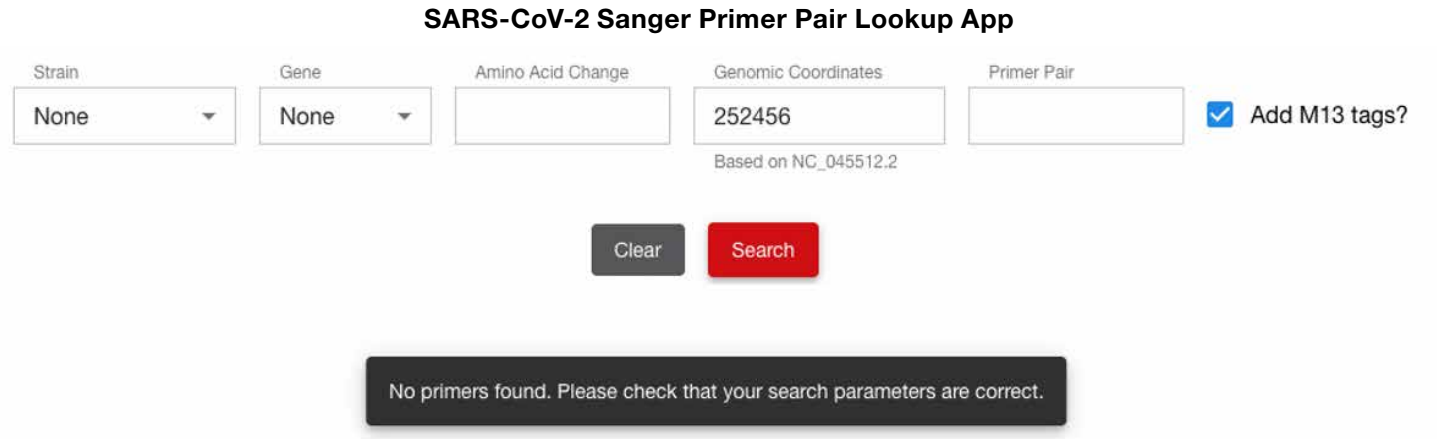

Figure 12. Flagging incorrect or unrecognizable entries. The database has a limited number of entries and lookup values available. Searches made against unmatched, incorrect, or unrecognizable values will trigger a notification. If this happens, check the entry to make sure it is correct.

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