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Targeted Sequencing by Sanger to Recover Key Mutations in Illumina SARS-CoV-2 Whole-genome Sequences

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1 *Works for me* This protocol is published without a DOI.

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ABSTRACT

The Spike (S) gene of SARS-CoV-2 demonstrates more frequent mutations in an otherwise conserved genome. With the proliferation of SARS-CoV-2 variants, there is an urgent need to continuously monitor the novel mutations arising to investigate viral diversity and vaccine escape mutants. Next-generation sequencing (NGS) technologiesthat are predominantly used to generate whole-genome sequences due to cost-effectiveness are prone to insufficient coverage and gaps in the sequence products. Sanger sequencing technology presents a complementary solution to enhance NGS sequencing through quick, gene-specific sequencing that has the potential to mitigate or avoid some of these challenges. Here we present sequencing primers that we designed and an associated workflow to recover key mutations (K417N, E484K, N501Y) in the receptor-binding domain of the 501Y.V2 lineage recently identified in South Africa.

The Sanger sequencing CE workflow

cDNA = complementary DNA; PCR = polymerase chain reaction; BDT = BigDye Terminator; BDX = BigDye Xterminator; CE = capillary electrophoresis

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Sanger, Spike, SARS-CoV-2, Targeted sequencing, Gaps, Whole Genome Sequencing (WGS)

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Sanger Sequencing 1h 34m

1h 34m

1 One-step RT-PCR

10m

1.1 On ice, prepare a master mix containing the components from Table 1 (add 10% extra for pipetting overage).

A	B
Table 1 – One-step RT-PCR Master Mix	
Component	Vol x1 (µl)
TaqPath 1-step RT-qPCR master mix (ThermoFisher Scientific)	5
501Y.V2 forward primer (5uM)*	1
501Y.V2 reverse primer (5uM)*	1
Nuclease-free water	3
Total	10

Table 1 - One-step RT-PCR Master Mix

* Primer details are shown in Table 2

A	B
Table 2 – PCR and sequencing 501Y.V2 primer details (accession: EPI_ISL_678597)	
Forward	GATCTCTGCTTTACTAATGTCTATGCAGAT
Reverse	GCTGGTGCATGTAGAAGTTCAAAAG
Amplicon Start	22698
Amplicon End	23098
Length	401 base pairs

Table 2 – PCR and sequencing 501Y.V2 primer details (accession: EPI_ISL_678597)

5m

1.2 In a 96-well plate, add 10 µl of master mix to each sample and no-template control (NTC) well.

15m

1.3 Add 10 µl of extracted RNA to each sample well. Mix gently by pipetting

2m

1.4 Add 10 µl nuclease-free water to the NTC well. Mix gently by pipetting

1.5 Seal the 96-well plate and briefly centrifuge the plate to collect the contents at the bottom of the wells. 2m

1.6 Place the 96-well plate on a thermocycler and run the PCR condition from Table 3. 1h

A	B	C	D
Table 3 – One-step Thermocycling Conditions			
Step	**Temperature (°C)**	**Time**	**Cycles**
UNG incubation	25	2 minutes	1
RT incubation	50	15 minutes	1
Enzyme activation	95	2 minutes	1
Amplification	95	3 seconds	40
60	30 seconds		
Hold	4		

2 PCR Purification 12m

2.1 In a new 96-well plate, add 10 µl of the PCR product to 4 µl Exo-SAP IT express reagent on ice. 5m

2.2 Mix by gentle pipetting, seal the plate and briefly centrifuge to collect the contents at the bottom of the wells. 2m

2.3 Run the following thermocycling conditions (Table 4). 5m

A	B	C
Table 4 - Exo-SAP Express IT Thermocycling conditions		
Step	**Temperature (°C)**	**Time**
Digest	37	4 minutes
Heat deactivation	80	1 minutes
Hold	4	hold

Table 4 - Exo-SAP Express IT Thermocycling Conditions

3 Cycle sequencing 1h 49m

3.1 On ice, prepare a master mix according to the components in Table 5, for each forward and reverse primer. 10m

A	B
Table 5 - BigDye™ Terminator cycle sequencing reaction	
Component	**Volume (µl)**
BigDye™ Terminator 3.1 Ready Reaction Mix	2
BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer	1
501Y.V2 forward OR reverse (3.2uM)	1
Nuclease-free water	5
Total volume	9ul

Table 5 - BigDye Terminator Cycle Sequencing Reaction

3.2 Add 9 µl of cycle sequencing master mix to each sample / NTC well of a 96-well plate. 10m

3.3 Add 1 µl of purified PCR product to each well. 5m

3.4 Mix gently by pipetting. 2m

3.5 Prepare an internal positive pGEM control by mixing the components from Table 6 and adding to a designated well of the 96-well plate. 2m

A	B
Table 6 – pGEM internal control reaction volume	
Component	Volume (µl)
BigDye™ Terminator 3.1 Ready Reaction Mix	4
M13 (-21) Primer	2
pGEM Control DNA	1
Deionized water	3
Total volume	10

3.6 Seal the plate, briefly centrifuge and place the plate in a thermocycler to run the conditions from Table 7. 1h 20m

A	B	C	D
Table 7 - Cycle sequencing thermocycling conditions			
PCR step	**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation	96	1 minute	1
Denaturation	96	10 seconds	25
Annealing	50	5 seconds	
Extension	60	4 minutes	
Hold	4	∞	1

Table 7 - Cycle sequencing thermocycling conditions

4 Cycle sequencing reaction purification

3h 19m

4.1

5m

Using the BigDye Xterminator Purification kit, prepare a master mix according to the volumes in Table 8. Prepare an additional 10% for pipetting overage.

A	B
Table 8 – BigDye XTerminator Mastermix	
Component	**Vol x1 (µl)**
SAM solution	45
XTerminator Solution	10
Total	55

Table 8 - BigDye XTerminator Mastermix

4.2

10m

Ensure that the solution is properly mixed and add 55 µl to each well.

4.3

32m

Seal the plate and vortex at 1800 rpm for 30 minutes.

4.4

2m

Centrifuge the plate at 1000 xg for 2 minutes.

4.5

2h 30m

Proceed with capillary electrophoresis on a DNA sequencer.