Identification of a putative functional domain in the E-viroporin of SARS-Cov-2

Bachelor thesis submitted in partial fulfillment of the requirements for the award of the degree of Bachelor of Biotechnology

الجامعة الألمانية بالقاهرة

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Submitted by:

Tamer ElSayed Abdelbary

49-1781

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Supervised by:

Dr. Ulrike Breitinger

Prof. Dr. Hans Breitinger

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Acknowledgments

I would like to express my sincere gratitude to all the people who have supported me throughout my academic journey. First and foremost, I would like to thank my supervisors, Dr. Ulrike Breitinger and Dr. Hans Breitinger, for there invaluable guidance, encouragement and feedback. Their passion and hard work will always inspire me to do the best I can in the future.

I am indebted to my research colleagues and friends at the B7.308 lab for their help and camaraderie.

I owe a special thanks to my family for their unconditional love and support throughout my life.

Abstract

The E-protein is one of two viroporins in SARS-Cov-2. It acts as an ion channel in the infected cells affecting the pH of many organelles and playing a critical role in virion assembly. Better understanding of the E-protein structure-function relationship is crucial for development of new drugs and treatments. In this thesis, a possible functional region of the E-Protein of SARS-Cov-2 suggested by bioinformatical methods was investigated by means of mutagenesis. 3 exchange mutations; L39K, T35F, and V29K were induced into the E-protein wildtype. These mutations were then compared to the wildtype by; MTT, western blot, and dot blot. The 3 mutations were successfully induced and investigated, and the results showed that mutations L39K and V29K were likely stabilizing and decreased the cytotoxic activity of the E-protein compared to that of the wildtype, while T35F did not show any significant difference compared to the wildtype in either activity of stability.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly transmissible and pathogenic coronavirus that emerged in late 2019 and has caused a pandemic of acute respiratory illness, coronavirus disease 2019 (COVID-19), worldwide. The virus is thought to have originated in bats and subsequently transmitted to humans[2]. SARS-CoV-2 is a positive-sense single-stranded RNA enveloped virus composed of a lipid bilayer and four structural proteins that drive viral particle formation. These four structural proteins are: the E and M proteins, which form the viral envelope; the N protein, which binds to the virus's RNA genome; and the S protein, which binds to human receptors[3].

SARS-CoV-2 encodes 29 proteins by its genome, several of which play critical roles in its replication and pathogenesis. Among these are the viroporins, E-Protein (also a structural protein as mentioned above) and ORF 3a, which have been shown to contribute to the massive release of inflammatory cytokines (cytokine storm) observed in patients with severe cases of COVID-19[4].

Viroporins are small, highly hydrophobic viral proteins that interact with membranes modifying the infected cell's permeability to ions or other small molecules. The name viroporin was coined after the discovery of proteins from several different types of viruses that share common characteristics. They are indispensable for viral replication, functioning as intracellular ion channels that disturb pH gradients of organelles and allow Ca^{2+} flux across ER membranes[4]. Viroporin subunit oligomerization is the general mechanism by which pores in the membrane are formed[5]. Viroporins also affect cellular functions, including the cell vesicle system and glycoprotein trafficking[6].

The E-protein or Envelope protein of SARS-CoV-2 is a 75-residue hydrophobic protein with an amino acid sequence nearly identical to that of SARS-CoV-1 E-protein[7]. Hexamethylene amiloride (HMA) and rimantadine have both been shown to block or diminish the activity of E-protein[7,8].

In this thesis, a possible functional region of the E-Protein of SARS-Cov-2 suggested by bioinformatical methods was investigated by means of mutagenesis.

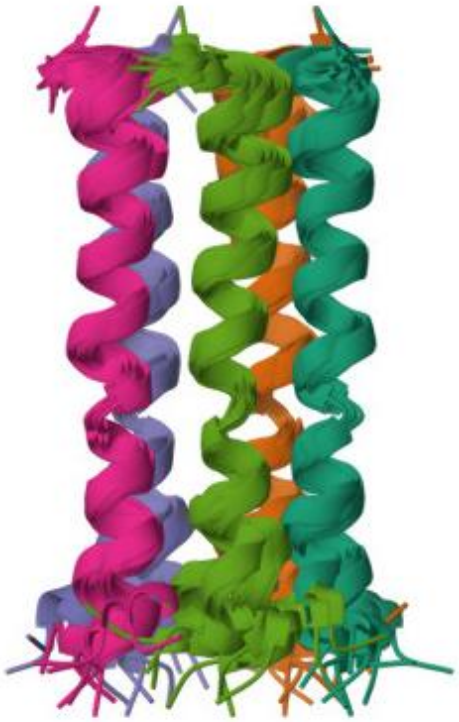

Figure 1.1: 3D view of E-protein pentamer in the membrane.

Materials

1. Laboratory Equipment:

Instrument	Manufacturer
Centrifuge 5810 R	Eppendorf, Germany
Cell culture hood suit AVC-4A1	ESCO, Singapore
C02 cell culture incubator	BINDER, Germany
DNA agarose gel electrophoresis chamber	Thermo Fisher Scientific, USA
Double beam spectrophotometer: V-630 UV/Visible spectrophotometer	JASCO, Japan
Floor incubator shaker	Eppendorf, Germany
Inverted light microscope	Carl Zeiss, Gemany
Laminar flow hood	BIOAIR, Italy
Microplate reader Wallac 1420 VICTOR 3	Perkin Elmer, USA
PCR machine T-personal thermocycler	Biometra, Germany
Pipettes	Carl Roth, Germany
Qubit fluorometer	Invitrogen, USA
Sensitive digital balance AUY 220	Shimadzu, Philippine
Single beam spectrophotometer 6305	JENWAY, UK
Thermomixer compact	Eppendorf, Germany
Vertical SDS gel electrophoresis chamber	Thermo Fisher Scientific, USA
UV gel imaging	Uvitec, UK
Vortex mixer- Reax top	Heidolph. Germany
Fluorescence Microscope	Zeiss, Germany

Table 1. 1: Laboratory Equipment

2. Laboratory materials:

Name	Manufacturer
12 well cell culture plate	Greiner Bio-one, North America
60 tissue culture plate	Greiner Bio-one, North America
Eppendorf reaction tubes (1.5, 2 ml)	Greiner Bio-one, North America
Cuvettes	Greiner Bio-one, North America
96 well plates	Greiner Bio-one, North America
PCR tubes	Sigma Aldrich, Germany
Polypropylen falcon tubes (15, 50 ml)	Greiner Bio-one, North America
Qubit assay tubes	Invitrogen, USA

3. Chemicals and reagents:

Chemical	Manufacturer
3 -(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2Htetrazolum-bromide (MTT)	SERVA, Germany
5-bromo-4-chloro-3-indolyphosphate (BCIP)	Carl Roth, Germany
Agar bacteriological	Thermo Fisher Scientific, USA
Agarose	Sigma Aldrich, Germany
Ammonium persulfate (APS)	Carl Roth, Germany
Ethidium bromide	Carl Roth, Germany
Nitro blue tetrazolium chloride (NBT)	Sigma Aldrich, Germany
Acrylamide Mix: bisacrylamide 40% (19: 1)	Ambion, USA
dNTPs	Carl Roth, Germany
DNA loading dye	Sigma Aldrich, Germany
GeneRuler 50bp DNA ladder	Thermo Fisher Scientific, USA
Dream Taq Master Mix (2X)	Thermo Fisher Scientific, USA
Ethanol absolute	El Nasr Pharmaceutical Chemicals, Egypt
Isopropanol	Thermo Fisher Scientific, USA
Methanol	SDFCL, Mumbai
N, N, N, N-Tetramethylendiamine(TEMED)	Thermo Fisher Scientific, USA

Table 1. 2: Laboratory materials

4. Enzymes:

Enzyme	Manufacturer
TaqI	Promega, USA
BstnI/MvaI	Fermentas USA
HinfI	Thermo Fisher Scientific, USA
BamHI	Promega, USA
PSTI	Thermo Fisher Scientific, USA
Dream Taq Green PCR Master Mix (2X)	Thermo Fisher Scientific, USA
FastAP alkaline phosphatase	Thermo Fisher Scientific, USA
HindIII	Promega, USA
Pfu polymerase	Thermo Fisher Scientific, USA
RNAse	Thermo Fisher Scientific, USA
T4 DNA Ligase	Thermo Fisher Scientific, USA

Table 1. 3: Enzymes

5. Primers:

Primer label	Description	Sequence	Size (bp)
p530	SARS E-Protein **L39K** FORWARD	CGCGCTTCGAAAGTGCGCGTATTGCTGCAACATTGTGAAC	40
p531	SARS E-Protein **L39K** REVERSE	GTTCACAATGTTGCAGCAATACGCGCACTTTCGAAGCGCG	40
p532	SARS E-Protein **T35F** FORWARD	GACACTGGCGATTCTGTTCGCGCTGCGC	28
p533	SARS E-Protein **T35F** REVERSE	GCGCAGCGCGAACAGAATCGCCAGTGTC	28
p534	SARS E-Protein **V29K** FORWARD	GGCGTTTGTGGTGTTTCTGCTGAAGACCCTGGCAATTCTG	40
p535	SARS E-Protein **V29K** REVERSE	CAGAATTGCCAGGGTCTTCAGCAGAAACACCACAAACGCC	40
P58	SARS E-Protein Flanking FORWARD	CACTTTGCCTTTCTCTCCACAGG	23
P59	SARS E-Protein Flanking REVERSE	CTGCAATAAACAAGTTGGGCCATG	24

Table 1. 4: Primers

6. DNA:

DNA Label	Description	Concentration
40a	Wildtype E-protein in pRK9 Vector	1 µg/µl
pRK9 BamHI-PstI	cut vector	1 µg/µl
GFP	GFP protein in pRK9 Vector	1 µg/µl
1.2 OE	L39K E-protein in pRK9 Vector	2.41 µg/µl
2.2 OE	T35F E-protein in pRK9 Vector	2.6 µg/µl
3.3 OE	V29K E-protein in pRK9 Vector	1.99 µg/µl

Table 1. 5: DNA

Methods

1. Bioinformatical search for functional sites in E-protein:

Multiple viroporin sequences of different viruses were obtained from the uniport[9] website and aligned using ncbi COBALT Constraint-based Multiple Alignment Tool[10]. In addition, the structure of the wildtype E-protein and its proposed mutants were investigated by helical wheel[11].

2. Primer design:

Three mutations were chosen based on the data obtained from the multiple alignments. The mutagenesis primers were designed to induce L39K, T35F, and V29K exchange mutations. In addition, a silent mutation was incorporated into each primer to add or remove a restriction site, thus allowing for test digests confirming the success or failure of the mutagenesis reaction. Table 2.1 shows the primers and what each silent mutation does, while figure 3.5 shows how the primers align to the wildtype vector. The silent mutation was designed with the help of software that I programmed which I named SMRF (Silent mutation for Restriction-site Finder). Snapshots of the use of this software as well as a website to download it from are present in the appendix. The primers were ordered and bought from biomers.net company.

Primer	Mutation induced	Restriction site added/removed
P530 & P531	L39K	Adds TaqI site
P532 & P533	T35F	Removes BstnI site
P534 & P535	V29K	Removes HinfI

Table 2. 1: Primer design in term of Restriction Site added/removed for each mutation

3. Overlap extension PCR part 1:

The first 2 PCR reactions are conducted as described by the diagram[12]:

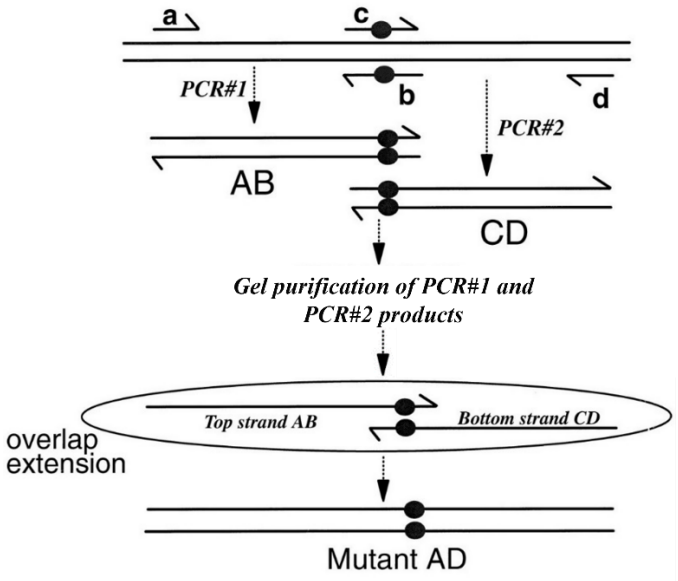

Figure 2. 1: Overlap Extension flow diagram

3.1. The PCR reaction mixtures were set up as follows:

PCR #1			
	Mutation 1 (L39K)	**Mutation 2 (T35F)**	**Mutation 3 (V29K)**
pfu Taq polymerase Master MIX	12.5 µl	12.5 µl	12.5 µl
p59 (reverse flanking primer)	2.5 µl	2.5 µl	2.5 µl
Forward mutagenesis primer	2.5 µl (p531)	2.5 µl (p533)	2.5 µl (p535)
40a DNA	0.5 µl	0.5 µl	0.5 µl
Water	7 µl	7 µl	7 µl

Table 2. 2: PCR #1 reaction mixture

PCR #2			
	Mutation 1 (L39K)	**Mutation 2 (T35F)**	**Mutation 3 (V29K)**
pfu Taq polymerase Master MIX	12.5 µl	12.5 µl	12.5 µl
p58 (forward flanking primer)	2.5 µl	2.5 µl	2.5 µl
Reverse mutagenesis primer	2.5 µl (p530)	2.5 µl (p532)	2.5 µl (p534)
40a DNA	0.5 µl	0.5 µl	0.5 µl
Water	7 µl	7 µl	7 µl

Table 2. 3: PCR #2 reaction mixture

3.2. The PCR reaction conditions were:

Thermocycler conditions			
Time	**Temperature**	**Step**	
1 min	96		Preheating
30s	98	23 cycles	Denaturing
1min	55		Annealing
2min	72		Elongation
5min	72		Final Elongation

Table 2. 4: Thermocycler settings for PCR #1, PCR #2, and PCR #3

4. PCR 3 product extraction using gel electrophoresis:

4.1. First a 1.5% agarose gel was cast and left to cure. The steps to do this are as follows:

4.1.1. 40 ml of TBE is added into a glass bottle and then 1.2 grams of agarose is added into the bottle. Next, the bottle is heated for 1:30 minutes in a microwave (or for however long it takes to dissolve all the agarose).

4.1.2. 40 ml of room temperature TBE is then added to the hot bottle once it comes out of the microwave. This step is to cool the agarose solution for the next step, which is to add 60µl of ethidium bromide (heat labile). The ethidium bromide acts as a pigment to allow us to visualize the double-stranded DNA under UV light.

4.1.3. Once the ethidium bromide has been added the solution is decanted into the electrophoresis mold with the comb and left to solidify for 20 minutes.

4.2. The products of the 1ST and 2rd PCR Reactions are then loaded onto the agarose gel while being divided into 2 areas. One with a low amount of the PCR product reaction mixture which I will henceforth call the sacrifice and one with the bulk of the product. This is done so that the gel could be cut up into two different regions after it runs, and the region with the low amount of the PCR reaction mixture (the sacrifice) can be visualized under mutation-inducing UV without damage to the other area containing the bulk of the DNA product. By doing this the location of the desired DNA bands can be obtained from the first half (the sacrifice) and then translated to the second half of the gel containing the bulk.

4.3. Once the samples are loaded, gel electrophoresis is run for 20 minutes at 120V-150V, and the gel is cut into the beforementioned areas. The sacrifice area is visualized under UV (Figure 2.2) and the locations of the bands are ascertained.

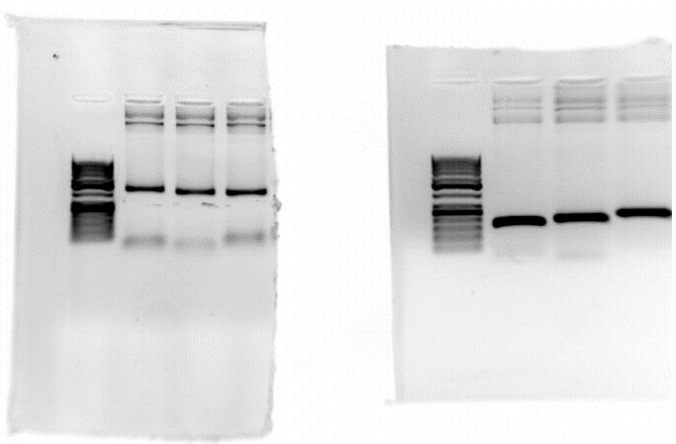

Figure 2. 2: The "Sacrifice area" under UV

4.4. The obtained locations of the bands are then translated to the second half containing the bulk and a thin cut directly under each band is done. This cut is then filled with folded filter paper and gel electrophoresis is again run for 5 minutes. As the electrophoresis is running the DNA band is then carried down into the filter paper and is absorbed/adsorbed into/onto it.

4.5. The filter paper is removed from the agarose gel and transferred into eppendorf tubes. The eppendorfs are spun down at 14000 rpm for 5 minutes and the now dry filter paper is thrown away. The remaining liquid at the bottom of the eppendorfs is the desired products from PCR#1 and PCR#2, which is used in the 3rd PCR reactions depicted in table 2.5.

5. Overlap extension PCR part 2:

5.1. The 3rd and final PCR reaction in the mutagenesis overlap extension was set up as follows:

PCR #3 (Overlap Extension)			
	Mutation 1	**Mutation 2**	**Mutation 3**
pfu Taq polymerase Master MIX	12.5 µl	12.5 µl	12.5 µl
p58 (forward flanking primer)	1.25 µl	1.25 µl	1.25 µl
p59 (reverse flanking primer)	1.25 µl	1.25 µl	1.25 µl
PCR #1 product	1.5 µl	1.5 µl	1.5 µl
PCR #2 product	1.5 µl	1.5 µl	1.5 µl
Water	7µl	7µl	7µl

Table 2. 5: PCR #3 reaction mixture

5.2. the conditions of the PCR reaction were the same as in 3.2.

6. *Ethanol precipitation of PCR#3:*

This step is done to clean the DNA from impurities such as enzymes and buffers which could affect future experiments.

6.1. 0.1 volume of 3M sodium acetate (NaOAc), pH 5.2 was added to the DNA to be precipitated.
6.2. 2.5 volumes of 100% ethanol (EtOH) or 0.7 volumes of isopropanol were then added and mixed gently by inverting.
6.3. The eppendorf was left to incubate for 10 minutes on ice and then centrifuged for 20 minutes at maximum speed (13,000 rpm or 12,000 g) before the supernatant being carefully aspirated out.
6.4. The pellet was washed once with 70% EtOH and vortexed to break up pellet, allowing the water access to salts that may be in the pellet.
6.5. The eppendorf is then centrifuged at maximum speed and the supernatant again removed.
6.6. The resulting pellet was Air-dried for 30 minutes using a heat block at 37 and resuspend in an appropriate volume of water.

7. *Determine DNA concentration using Qubit*[13]*:*

7.1. First the fluorometer Is calibrated every once in a while, using the standards supplied.
7.2. Once the fluorometer is calibrated, the Qubit™ working solution is prepared by diluting the Qubit™ reagent 1:200 in Qubit™ buffer. 200µL of working solution is needed for each standard and sample.
7.3. The sample and standard are prepared according to the following Table 2.6:

	Standard assay tubes	User sample assay tubes
Working solution† (from step 2)	190 µL	180–199 µL
Standard (from kit)	10 µL	—
User sample	—	1–20 µL
Total Volume in each assay tube	200 µL	200 µL

Table 2. 6: Qubit measuring mixtures

7.4. all tubes are vortexed for 2–3 seconds.
7.5. the tubes are incubated for 2 minutes at room temperature.
7.6. Then the tubes are inserted into the Qubit™ Fluorometer and the readings are taken with the broad range ds-DNA protocol.

8. *Cutting mutated DNA and vector with BamHI and PstI:*

8.1. The cutting with BamHI and PstI is done both on the vector and on the mutated DNA created in the previous steps.

8.2. The cutting, regarding the mutated DNA, was accomplished with the reaction mixture (Table 2.7):

	Volumes
DNA	16.5 µl
10x FD buffer	2 µl
BamHI	1 µl
PstI	0.5 µl

Table 2. 7: The reaction was set at 37°C for 40 minutes.

8.3. In this case the vector was obtained already cut and ready for the next step

9. Ligation:

9.1. First ethanol precipitation is done on the digestion product as described in step 6. This is done so that the restriction enzymes from step 8 are disabled, and the buffer condition is removed.

9.2. Next Equation 1 is used to calculate the amount of insert (cut mutated DNA) to use in the ligation reaction.

Equation 1 $$\frac{ng\ vector \times kb\ size\ of\ insert}{kb\ size\ of\ vector} \times Molar\ ratio\ of\ insert{:}\ vector = ng\ of\ insert$$

9.3. The Parameters chosen for the ligation were:

ng vector = 40 ng kb size of vector = 4.71

kb size of insert = 0.562 Molar Ration = 4

9.4. Once the ng of the insert needed was calculated the ligation reaction was completed according to the following reaction mixture (Table 2.8):

	Volume/ng amount
pRK9 BamHI – PstI	40 ng
10x T4 DNA Ligase Buffer	0.7 µl
T4 DNA Ligase	0.7 µl
Insert	Calculated amount
Water	Ad 7 µl

Table 2. 8: Ligation reaction mixture

9.5. The ligation was done for 10 minutes at room temperature.

10. Transformation (XL1-Blue):

10.1. XL1-Blue competent cells are thawed on ice (ca. 15 min)

10.2. 5 µl ligation reaction is added to the cells (~45 µl bacteria) in an eppendorf.

10.3. The contents of the eppendorf are mixed very gently,

10.4. The eppendorf is incubated in ice for 30 minutes, then transferred to a water bath at 42ºC for 1 minute.

10.5. The cells are then incubated on ice for 2 minutes.

10.6. After the 2 minutes, ~120 µl LB medium was added, and the cells were left to shake at 37 ºC for 1 h at 225-250 rpm in the thermomixer.

10.7. 80 to 150 µl were then taken from the eppendorf and plated on agar containing antibiotics (ampicillin).
10.8. In addition to the 3 mutations 3 controls were also transformed and plated:

Negative Control:	only water was added in the transformation.
Positive Control:	42a (previously tested ampicillin-resistant) vector was transformed.
Negative Control:	cut vector without insert (unligated/linear) was transformed.

10.9. The plates are then transferred to the incubator overnight and 4 colonies for each mutation were selected in the morning.

11. DNA Minipreparation:

This step is done to obtain a higher amount of DNA with relatively high purity.

11.1. First the selected colonies are extracted from the plate using a loop or a pipette tip.
11.2. For each mutation 4 colonies were selected in the hope that at least one colony per mutation contained the correct mutated vector.
11.3. The bacteria colonies were transferred to a test tube in 2 ml LB containing ampicillin and left to grow at 37 ºC, slowly shaking, overnight.
11.4. 1.5 ml of overnight culture was then transferred into an Eppendorf tube and centrifuged (15.000 rpm, RT, 5 min)
11.5. The remaining bacteria solution was kept in the fridge until positive results from Sequencing.
11.6. Once the centrifugation was complete the SN was discarded into the autoclave waste and the pellet was resuspended in 225 µl Buffer 1(Table 2.9).
11.7. Next, 225 µl of freshly prepared buffer 2 (Table 2.9) is added, and everything is mixed gently by inversion 4-6 x.
11.8. The eppendorfs are then Incubate for 3 min at RT.
11.9. 380 µl of buffer 3 (Table 2.9) is added next, and again everything is mixed gently by inversion 4-6 x.
11.10. The eppendorfs are centrifuged (4ºC, 15000 rpm, 5 min).
11.11. 700 µl of the Supernatant was transferred into a new Eppendorf tube and 700 µl Isopropanol was added to this eppendorf.
11.12. The old eppendorf was discarded in the waste and the new one was mixed well and incubated for 10 min on ice.
11.13. Centrifugation again (15000 rpm, 5 min). And this time we discard the supernatant and left the pellet.
11.14. The pellet was washed with 500 µl 70 % Ethanol.
11.15. Centrifugation again is done (15000 rpm, 5 min) and the supernatant was discarded.

11.16. The pellet was air-dried at 37 ºC and the DNA was dissolved in an appropriate volume of Water once the pellet was dry.

	Component	Final Concentration in Buffer
Buffer 1 (Cell resuspension Solution)	Tris (pH 7.5)	50 mM
	EDTA (pH 8.0)	10 mM
	RNAse A(fresh)	0.1 mg/ml
Buffer 2 (Cell Lysis Solution)	NaOH	0.2M
	SDS	1%
Buffer 3 (Neutralization Solution)	Guanidine hydrochloride (pH 4.8)	4.09M
	KOH	0.759M
	Acetic acid	2.879M

Table 2. 9: Buffer components used in mini and midi preparations

12. Test Digest (test for insert):

12.1. The presence of the insert in the vector can be tested by cutting the obtained DNA from the miniprep with restriction enzymes flanking the insert. The two I chose for this are BamHI and HindIII.

12.2. The cutting was carried out according to the following reaction mixture:

	Volume
10x Fast digest Buffer	1 µl
BamHI	0.2 µl
HindIII	0.2 µl
DNA (from miniprep)	2 µl (2-6 µg DNA)
Water	6.6 µl

Table 2. 10: The digestion was carried out at 37°C for 30 minutes.

12.3. After 30 minutes the samples were loaded onto a gel created as described in step 4.1 with the addition of the ladder (Figure 2.3) and run for 30 minutes.

12.4. The gel was then visualized using UV light.

12.5. The wells that contained the insert Should have a clear band at 550 bp size region.

12.6. One of the insert-positive wells were chosen for each mutation.

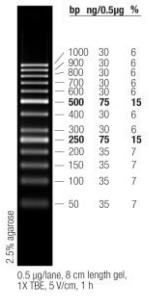

Figure 2. 3: GeneRuler

13. Test Digest (test for mutation):

13.1. The mutagenesis reaction product can now be tested since all the previous steps should have resulted in a minipreparation containing DNA with the desired mutation. To do this, utilization of the silent mutation/restriction site was used. If the mutagenesis reaction was truly successful, then digestion of the wildtype and the mutant with the respective restrictive enzyme for each mutant would show a clear difference between the two. As can be seen in Figure 3.7.

13.2. The reaction mixture for the digestion of the 3 mutations was as follows:

Mutation L39K		Mutation T35F		Mutation V29K	
	Volume		Volume		Volume
10x BufferE	1µl	**10x BufferR**	1µl	**10x Green Buffer**	1µl
TaqI	0.4 µl	**MavI**	0.4 µl	**HinfI**	0.4 µl
water	6.6 µl	**water**	6.6 µl	**water**	6.6 µl
DNA(from Miniprep)	2 µl (2-6 ug DNA)	**DNA(from Miniprep)**	2 µl (2-6 ug DNA)	**DNA(from Miniprep)**	2 µl (2-6 ug DNA)

Table 2. 11: Digestion reaction mixtures for the 3 mutations

13.3. The reaction was incubated at the optimum temperature and time for each enzyme.
13.4. Once the digestion was complete, loading dye was added to the samples, and the samples were then loaded into a gel created as in step 4.1.
13.5. The gel was run for 25 minutes and then visualized under UV.

14. DNA Midipreparation:

14.1. This step is the same as step 11 but with a higher volume of LB used for a 50 ml tubes. Therefore, there is more bacteria grown and there is more DNA That can be extracted and concentrated.

15. Sequencing

15.1. The following sequencing mixture is prepared and sent to LGC genomics in Germany:

	Amount/Volume
DNA (each of the 3 mutations)	1.4 µl
P58 (Primer)	20 picomol
Water	Ad 14 µl

Table 2. 12: Sequencing preparation

16. Transfection of HEK 293 Cells:

16.1. First the cells were passaged from a confluent 10 cm plate into five 1.5cm plates and left in the incubator for 1 day.
16.2. The next day the cells in each of the five plates were transfected with a different DNA: the 3 mutations (L39K, T35F, and V29K) in pRK9, 40a plasmid (wildtype E-protein in pRK9), and GFP (transfection positive control).
16.3. The transfection was carried out as follows:

16.3.1. Two eppendorfs are prepared for each transfection. The contents of each eppendorf are as follows (Table 2.13):

	Component	**Volume/Amount**
Eppendorf tube 1	Transfection DNA	3 µg
	DMEM	500 µl
Eppendorf tube 2	PEI (1µg/µl)	50 µl
	DMEM	450 µl

Table 2. 13: The two tubes mixtures used in transfection

16.3.2. The content of eppendorf 2 is then added to eppendorf 1 and mixed by pipetting up and down 3 times.
16.3.3. The transfection mixture is left to incubate for 20 minutes at room temperature.

16.3.4. Once the incubation time is over the transfection mixture is added to the cell culture plate drop by drop while simultaneously swirling the plate (to spread the transfection medium evenly over the plate).

16.4. Next the plates were placed in the incubator for 2 days to express the vectors which were transfected.

16.5. The plates were then inspected under the microscope and the GFP plate was tested for cell fluorescence. If the transfection was successful, some of the cells in the GFP plate would appear green.

16.6. After the transfection was confirmed to be successful the medium from each plate was removed and the plates were wrapped with parafilm before being put in the -20°C until step 18 (membrane preparation).

17. SDS-PAGE gel (preparation):

17.1. The SDS gel allows for the separation of different proteins in a mixture into different bands. This is then subsequently transferred to a membrane and immunostained.

17.2. The SDS gel was prepared according to the following steps:

17.2.1. Carefully build up the glass plates as in figure 2.4 and seal them with 1.5% agarose.

17.2.2. Prepare the separating gel according to Table 2.14 and pour it into the gap between the plates, add 0.5 ml isopropanol on top.

17.2.3. After the separating gel is polymerized, prepare the stacking gel according to table 2.14. Next: Remove isopropanol, wash with water 2-3x, pour the stacking gel, and insert the comb.

17.2.4. If the gel is not needed immediately, you can wrap it in wet tissue and cellophane foil and keep it in the fridge for several weeks.

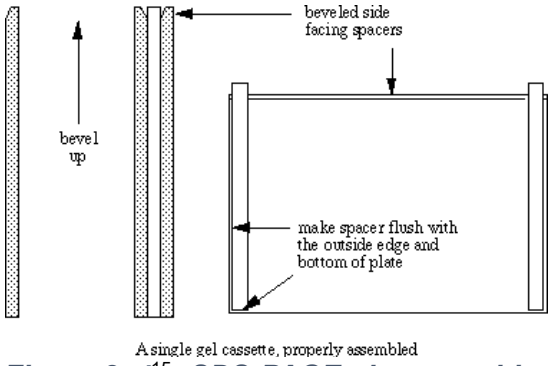

Figure 2. 4[15]: SDS-PAGE glass mould setup

Separating gel (12% acrylamide)	**Volume**	**Stacking gel (4% acrylamide)**	**Volume**
30 % Acrylamide	2.4 ml	**30 % Acrylamide**	335 µl
1 M Tris pH 8.8	2.25 ml	**2.5 M Tris pH 6.8**	125 µl
H_2O	1.25 ml	**H_2O**	2 ml
10 % SDS	60 µl	**10 % SDS**	25 µl
10 % APS	30 µl	**10 % APS**	25 µl
TEMED	3.8 µl	**TEMED**	2.5 µl
Bromophenol blue	0	**Bromophenol blue**	2.5 µl
Sum	6 ml	**Sum**	2.5 ml

Table 2. 14: Stacking and Seperating gels components

18.Membrane preparation (1):

18.1. The membrane proteins from the transfected cells made in step 16 must be isolated and prepared for SDS-PAGE Run.
18.2. First the plates were removed from the freezer and the cells were detached using 1.5 ml ice-cold PBS buffer and transferred to eppendorfs.
18.3. Next, the cells were centrifuged at 2000 x g for 10 minutes.
18.4. The Supernatant was then decanted, and the cell pellet was resuspended in 5 volume parts (minimum 2 ml) of Buffer H.
18.5. The eppendorf was then kept on ice for 5 minutes.
18.6. The cells were broken down and homogenized using a glass cell homogenizer.
18.7. The protein suspension was centrifuged at 17000 rpm for 20 minutes and the supernatant was decanted.
18.8. The pellet containing the membrane fraction was resuspended in buffer B (2 volume parts)
18.9. Bradford Protein Assay was performed to determine the protein concentration and all protein solutions were set to a concentration of 1 µg/µl.
18.10. Next, 5x SDS loading buffer (Table 2.15) was added to the membrane fraction, and the eppendorf was heated at 99°C for 1 minute. This step denatures the membrane proteins and adds a negative charge to them.

SDS Loading buffer (5x)	
	Concentration
Tris pH 6,8	0.313 M
Glycerine	50%
SDS	10%
Bromophenol blue	0.05%
DTT	0.5 M

Table 2. 15: SDS Loading buffer (5x) components

18.11. The protein solution was now ready to run on SDS-PAGE.

19. Membrane preparation (2):

Due to the highly unstable nature of the small-sized E-protein a second membrane preparation protocol was tested in an attempt to decrease the degradation and increase the E-protein yield.

19.1. Transfected cells from the plates created in step 16 were detached using 400 µl of PBS + PMSF (1mM) and transferred to an eppendorf tube.
19.2. The eppendorf was centrifuged 2000 x g for 10 minutes and the supernatant was decanted.
19.3. The pellet was resuspended in 100 µl PBS + protease inhibitor (~1 µg/µl)
19.4. Bradford Protein Assay was performed to determine the protein concentration and all protein solutions were set to a concentration of 1 µg/µl.
19.5. Next, 5x SDS loading buffer (Table 2.15) was added to the membrane fraction, and the eppendorf was heated at 99°C for 1 minute. This step denatures the membrane proteins and adds a negative charge to them.
19.6. The protein solution is now ready to run on SDS-PAGE.

20. SDS-PAGE (Run):

20.1. First the SDS-PAGE gel was inserted into the electrophoresis apparatus and running buffer (1x made from dilution of 10x in Table 2.16) was added to cover the gel.
20.2. The protein marker was then loaded into the second lane.
20.3. Next, the samples were loaded into the wells while avoiding spillover to other lanes.
20.4. The gel was run at 120 V until the protein left the stacking gel (~ 20 minutes) and then the voltage was increased to 150 V for the separating gel.

Running buffer (10x) 1 liter		
	Volume/Amount	**Concentration**
Tris-Base	30.3 g	0.25 M
Glycine	144 g	1.924 M
SDS	10 g	0.03467 M
Water	ad 1 liter	

Table 2. 16: Constituants of 1 littre of SDS-PAGE Running buffer (10x)

20.5. The gel was left to run until the bromophenol front reached the end of the gel as can be seen in Figure 2.5.

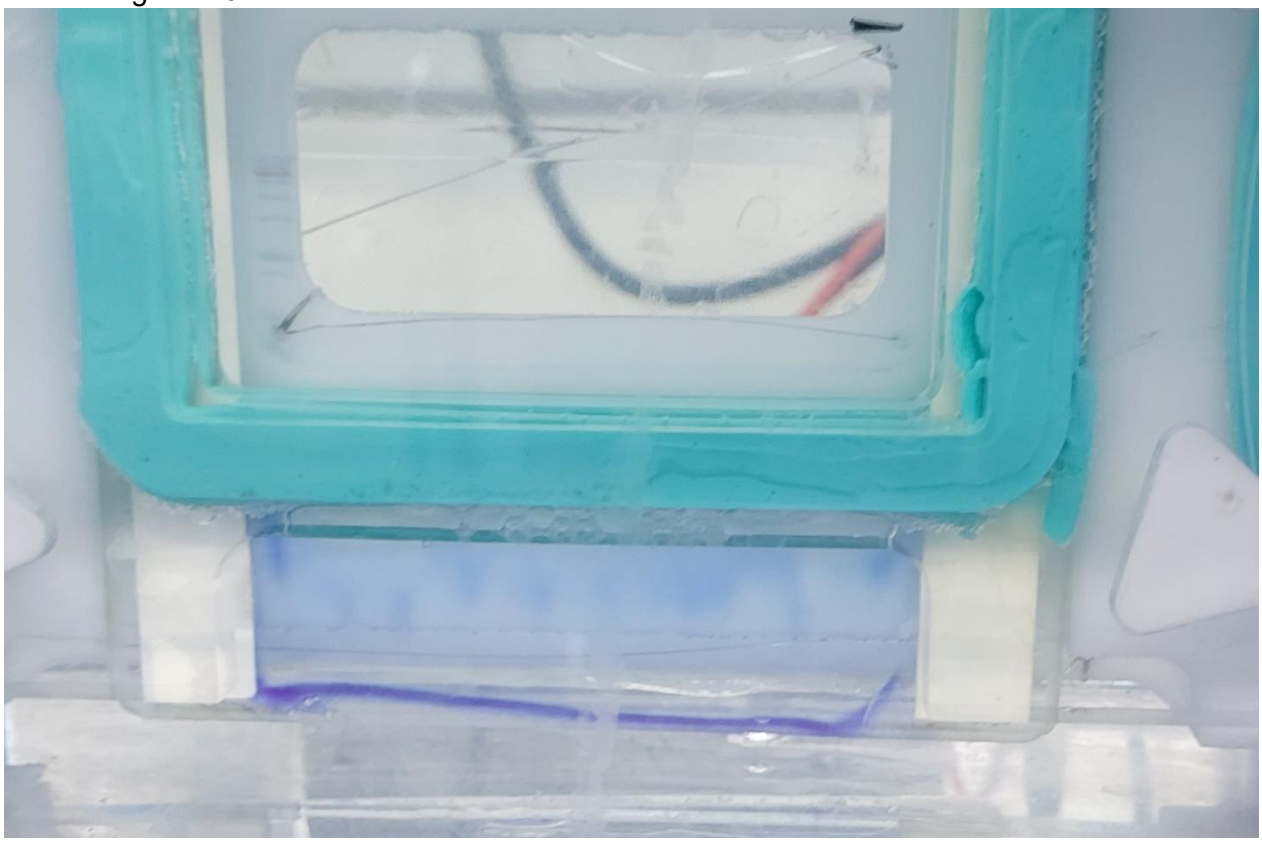

Figure 2. 5: Image showing the bromophenol front reaching the end of the gel indicating that the SDS-PAGE run can now be stopped

21. Electroblotting (Western blotting part 1):

In this step, the protein bands were transferred from the SDS-PAGE gel onto a PVDF membrane to allow for immunostaining.

21.1. First 6 pieces of filter paper were incubated in Towbin buffer (Table 2.17).

Towbin buffer	
	Concentration
Tris	25 mM
Glycerine	192mM
Methanol	20%

Table 2. 17: Towbing buffer constituants

21.2. Next, the PVDF membrane was conditioned by doing the following:
- 21.2.1. Fist, the membrane is incubated in 100% methanol for 15 seconds.
- 21.2.2. Then, 2 minutes in water
- 21.2.3. And finally, 5 minutes in Towbin buffer

21.3. The Western blot transfer was organized as follows in the electroblotting apparatus:

Lid
3 filter paper soaked in Towbin buffer
PVDF membrane
acrylamide gel (without stacking gel)
3 filter paper soaked in Towbin buffer
Bottom

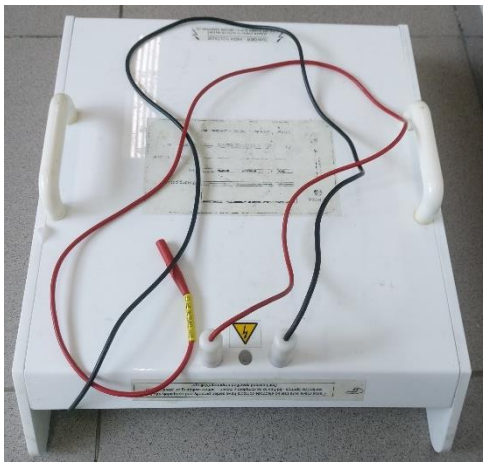

Figure 2. 6: Electroblotting apparatus

21.4. Then air bubbles were removed carefully!

21.5. The transfer was then started and left to run for 60 minutes at 150mA constant current and room temperature.

22. Immunostaining (Western blotting part 2):

22.1. Now that the protein bands had been transferred to the PVDF membrane. The next step was to visualize the bands by immunostaining.

22.2. To do this first, unspecific binding sites on the PVDF membrane were blocked by adding TBB buffer for 30 minutes with slow shaking.

22.3. The TBB buffer was then discarded and mouse anti-myc-tag antibodies (primary antibody) in TBB were added and left on the membrane for 60 minutes with shaking.

22.4. Then the antibody solution was poured into a 15 ml falcon tube for reuse.

22.5. The blot was washed using (1) TBS, (2) TWS, and (3) TBB buffer (Table 2.18) (each for 5 minutes) to remove any unbound primary antibody and prepare the membrane for the secondary antibody.

22.6. Anti-mouse bound to alkaline phosphatase (AP) antibodies (secondary antibody) were then added to the membrane and left to shake for 30 minutes.

22.7. Then the antibody solution was again poured into a 15 ml falcon tube for reuse.

22.8. The blot was washed using (1) TBS, (2) TWS, and (3) TBB buffer (Table 2.18) (each for 5 minutes) to prepare it for development.

TBS buffer (1x)		TWS buffer (1x)		TBB buffer (1x)	
	Concentration		**Concentration**		**Concentration**
Tris-Base pH8.0	50 Mm	**Tris-HCl pH8.0**	50 Mm	**Tris-HCl pH8.0**	50 Mm
NaCl	150 mM	**NaCl**	150 mM	**NaCl**	150 mM
		TritonX-100	0.5%	**TritonX-100**	0.5%
				BSA	3%

Table 2. 18: The constituants of the different buffers used in Immunostaing

23. Blot development (using BCIP/NBT)

23.1. First BCIP and NBT stock solutions were prepared according to Table 2.19.

substrate buffer		NBT (1ml)		BCIP (1ml)	
	Concentration		**Amount**		**Amount**
Tris-HCl pH9.5	100 Mm	**NBT**	30 mg	**BCIP**	15 mg
NaCl	100 mM	**70% DMF**	1 ml	**Water**	1 ml
$MgCl_2$	5 mM				

Table 2. 19: Composition of buffer and chemical dilutions used in Blot Development.

23.2. Prior to use, 100 µl NBT stock and 100 µl BCIP stock were added to 10 ml of Substrate Buffer, mixed, and immediately added to the blot.
23.3. The blot was then left on the shaker to observe band development.
23.4. When the development was sufficient and before background clouds started to appear, the reaction was quenched by washing multiple times with water.
23.5. The bands were now visible, having a purple colour.

24. MTT

In this experiment, the toxic effect of Wildtype E-protein and the 3 mutations created on eukaryotic HEK 293 cells were investigated with and without Rimantadine (E-protein blocker). This was done by transfecting the cells with vectors expressing said proteins.

24.1. The first step in this experiment was to seed a 96 Well plate with HEK 293 cells. This was done by doing the following:
  24.1.1. A 10 cm plate was harvested in 4 ml DMEM, and the cell concentration was calculated using a hemocytometer.
  24.1.2. From the calculated cell concentration, the appropriate volume of the cell solution was taken and added to 10 ml of DMEM to give a final concentration of 14,000 cfu/120 µl.
  24.1.3. Next, 120 µl (14,000 cells) were added to each well.
24.2. The plate was then left in the incubator to grow for 1 day.
24.3. The next day the MTT plate was transfected in the same manner as in step 16. But in this case, an appropriate volume of transfection medium was prepared for the number of wells. Then 15 µl of the transfection medium was added per well to give a final volume of 135 µl per well. A layout of the well and the calculations used for the transfection can be found in the Appendix.

24.4. 4.5 µl of rimantadine 30x (1500 µM) was added to the required wells to give a final concentration of 50 µM per well.
24.5. The 96-well plate was then placed in the incubator for 4 days for the cells to express the transfected vectors.
24.6. After the 4 days, the medium in the wells was removed and the cells were washed with PBS.
24.7. 100 µl MTT made up in water to a final concentration of 0.5 mg/ml was added to each well.
24.8. It was then left in the Incubator for 30-59 minutes at 37°C, until intracellular purple formazan crystals were visible under the microscope.
24.9. The MTT solution was removed and 100 µl of DMSO was added.
24.10. The DMSO was left on for 30 minutes at room temperature to lyse all the cells and release the intracellular formazan purple crystals to solution.
24.11. Finally, the plate was put in a Wallac 1420 VICTOR 3 Microplate reader and the absorbance at 595 for one second was recorded.

25. Dot blot:

In this assay, the protein of interest was blotted on a PVDF membrane as in a western blot but in this case, no separation of the proteins is done by SDS-PAGE. The advantage of this assay is that it is quick and easy, but it does not give any information on the size of the protein. The only information it gives is whether the protein is present or absent and the relative amount between different samples (higher/Lower concentration).

25.1. Mouse Anti-Myc-tag antibody (primary antibody) is added to transfected cells in a 24-well plate, created as described in step 16, but this is done while the cells are still alive and intact.
25.2. The antibody is left on for 60 minutes while the cells are in the incubator.
25.3. After that, the plates were washed 3x with PBS making sure not to detach the cells.
25.4. Next, Anti-mouse bound to alkaline phosphatase (AP) antibody (secondary antibody) was added.
25.5. The antibody was left on for 30 minutes while the cells were in the incubator.
25.6. And again, the plates were washed 3x with PBS making sure not to detach the cells.
25.7. The cells were detached into 100 µl of PBS and the protein concentration was determined using the Bradford assay.
25.8. All protein solutions were then set to the same concentration.

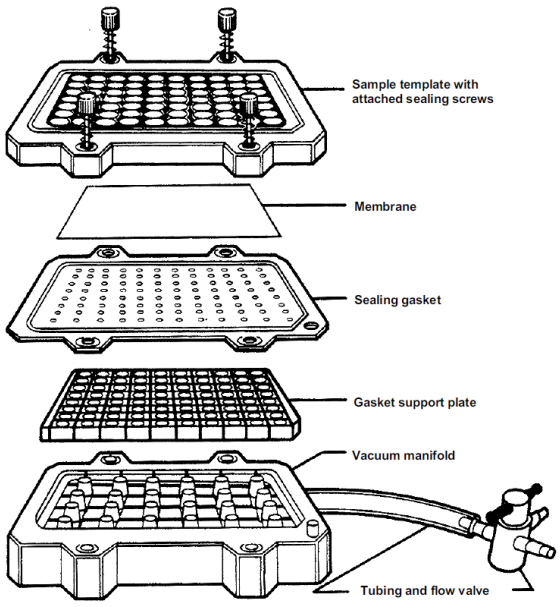

Figure 2. 7[16]: Demonstration of the layout of the Dot blot apparatus

25.9. Next, the dot blot apparatus was assembled as seen in figure 2.7 and Figure 2.8. And 15 µg and 40 µg of protein were each added in triplicates to the wells quickly.
25.10. The pump was then turned on. And the proteins were sucked onto the membrane.
25.11. All wells were washed with 15 µl PBS.
25.12. And finally, the blot was developed using BCIP/NBT as described in step 23.

A picture of the developed blot was taken and analysed to determine the relative concentrations of the dots using ImageJ[13].

Figure 2. 8: Photo of the completed Dot blot apparatus

Results and Discussion

1. Bioinformatical search for functional sites in E-protein:

The search resulted in the alignment seen in Figure 3.1, demonstrating the presence of a conserved region. The helical wheel of each theoretical wheel can be seen in Figure 3.1.

Accession	Description
lcl|Query_10001	sp|P33515|POLG_MCFA|279-705 OS=Mosquito cell fusing agent OX=31658 PE=1 SV=1
lcl|Query_10002	sp|P33515|POLG_MCFA|1328-1451 OS=Mosquito cell fusing agent OX=31658 PE=1 SV=1
lcl|Query_10003	sp|P14403|POLG_JAEVN|147-222 OS=Japanese encephalitis virus (strain Nakayama) OX=11076 PE=1 SV=1
lcl|Query_10004	sp|P14403|POLG_JAEVN|1302-1432 OS=Japanese encephalitis virus (strain Nakayama) OX=11076 PE=1 SV=1
lcl|Query_10005	sp|Q7T6D2|POLG_OHFV|206-280 OS=Omsk hemorrhagic fever virus OX=12542 PE=1 SV=1
lcl|Query_10006	sp|Q7T6D2|POLG_OHFV|1359-1489 OS=Omsk hemorrhagic fever virus OX=12542 PE=1 SV=1
lcl|Query_10007	sp|C8XPA8|POLG_BANV|198-272 OS=Banzi virus OX=38837 PE=3 SV=1
lcl|Query_10008	sp|C8XPA8|POLG_BANV|1341-1469 OS=Banzi virus OX=38837 PE=3 SV=1
lcl|Query_10009	sp|C8XPB2|POLG_EHV|207-281 OS=Edge Hill virus OX=64296 PE=3 SV=1
lcl|Query_10010	sp|C8XPB2|POLG_EHV|1352-1480 OS=Edge Hill virus OX=64296 PE=3 SV=1
lcl|Query_10011	sp|P27958|POLG_HCV77|747-809 OS=Hepatitis C virus genotype 1a (isolate H77) OX=63746 PE=1 SV=3
lcl|Query_10012	sp|Q32ZD7|POLG_ILHV|211-285 OS=Ilheus virus OX=59563 PE=3 SV=1
lcl|Query_10013	sp|Q32ZD7|POLG_ILHV|1367-1497 OS=Ilheus virus OX=59563 PE=3 SV=1
lcl|Query_10014	sp|Q91B85|POLG_ALKV|207-281 OS=Alkhumra hemorrhagic fever virus OX=172148 PE=1 SV=1
lcl|Query_10015	sp|Q91B85|POLG_ALKV|1361-1491 OS=Alkhumra hemorrhagic fever virus OX=172148 PE=1 SV=1
lcl|Query_10016	sp|Q6DV88|POLG_YEFVA|211-285 OS=Yellow fever virus (strain Ghana/Asibi/1927) OX=407134 PE=1 SV=1
lcl|Query_10017	sp|Q6DV88|POLG_YEFVA|1355-1484 OS=Yellow fever virus (strain Ghana/Asibi/1927) OX=407134 PE=1 SV=1
lcl|Query_10018	sp|Q32ZD5|POLG_KOKV|212-286 OS=Kokobera virus OX=44024 PE=1 SV=1
lcl|Query_10019	sp|Q32ZD5|POLG_KOKV|1363-1492 OS=Kokobera virus OX=44024 PE=1 SV=1
lcl|Query_10020	sp|P14335|POLG_KUNJM|216-290 OS=Kunjin virus (strain MRM61C) OX=11078 PE=1 SV=1
lcl|Query_10021	sp|P14335|POLG_KUNJM|1375-1505 OS=Kunjin virus (strain MRM61C) OX=11078 PE=1 SV=1
lcl|Query_10022	sp|D7RF80|POLG_KFDV|207-281 OS=Kyasanur forest disease virus OX=33743 PE=1 SV=1
lcl|Query_10023	sp|D7RF80|POLG_KFDV|1361-1491 OS=Kyasanur forest disease virus OX=33743 PE=1 SV=1
lcl|Query_10024	Sars-Cov-2 E protein/None/
lcl|Query_10025	Ebolavirus/none/
lcl|Query_10026	InfluenzaM2//
lcl|Query_10027	MERSE//
lcl|Query_10028	SARS-1E1//
lcl|Query_10029	HCVP71a//
lcl|Query_10030	HCVP72a//
lcl|Query_10031	HCVP73a//
lcl|Query_10032	HCVP74a//

```
Query_10001  1    [341]STSVSIECALNPISQGWRLARHVVDRYRRFGVSGVAGVWQDLVGKFSVGAFFSNTALLVILVLAALIDKRIAFLL  416
Query_10002  1    [ 50]IPDLKGRYGEDGIVVGAASSSGYLPELVFVFLLGFAVTSTSYFLGALYLLIATSTNLPVVIIRMLRMKLTASNR-  124
Query_10003  1         SVSVQTHGESSLVNKKEAWLDSTKATRYLMKTENWIVRNPGYAFLAAILGWMLGSNNGQRRWYFTILLLLVAPAY  75
Query_10004  1    [ 58]DISWEMDAAITGSSRRLDVKLDDDGDFHLIDDPGVPWKVWVLRMSCIGLAALTPWAIVPAAFGYWLTL--KTTKR  131
Query_10005  1         SVLIPSHAQKDLTGRGQRWLEGDTIRSHLTRVEGWVWKNKSLTLAVVVIVWMTVESAVTRI-VIVSALLCLAPAY  74
Query_10006  1    [ 56]CVEWNPDLADEGGEISLRVRQDALGNFHLTELEKEERMMAFWLLAGLTASALHWTGILVVMGLWTMSEMLRSARR  131
Query_10007  1         AVVITAHLDQGLTTKKETWLGSSHFETQVQKVEKWIIRNPTYAIAAILMSWYIGNSLKQRV-VLLLLTLALGPAY  74
Query_10008  1    [ 57]DISWSDEAVVSGEARRFDVALNDSGEFKLLDEPPVSWLNVSFLVVAIVASSLHPIALVVTLVAWTYWR---TEKR  129
Query_10009  1         AAVITAHIDKGLTTRQEKWLSTSMGERQIQRIERWMMRNPFYAAISLLLAWWVGSDIKQKV-LIAFLVLAIGPAY  74
Query_10010  1    [ 57]DITWEEEAVHSGSSTRYDVTLNEAGEFKLVHEEPVVWSHVVFLVVALIAASVHPIALVVVTIIWTYGK---KHLR  129
Query_10011  1         ----------------ALENLVILNAASLAGTHGLVSFLVFFCFAWYLKGRWVPGAVYALYGMWPLLLLLLALPQ  59
Query_10012  1         AINIPHHGESHLENRATPWMDTTKTTKYLTKVENWVIRNPGYALVALATAWMLGSNTPQRV-VFMIMMMLIAPAY  74
Query_10013  1    [ 58]DISWINEAEITGTSPRLDVELDSNGDFKMINDPGVPMWMWTCRMGLMAMAAYNPVLIPVSMAGYWMTV--KIHKR  131
Query_10014  1         SVSIPVHAHSDLTGRGHKWLRGDSVKTHLTRVEGWVWKNKLLTMAFCAVVWMVTDSLPTRF-IVITVALCLAPTY  74
Query_10015  1    [ 56]EVEWNPDLVNEGGEVNLKVRQDAMGNLHLTEVEKEERAMALWLLAGLVASAFHWAGILIVLAIWTFFEMLSSGRR  131
Query_10016  1         AIDLPTHENMGLKTRQEKWMTGRMGERQLQKIERWLVRNPFFAVTALTIAYLVGSNMTQRV-VIALLVLAVGPAY  74
Query_10017  1    [ 57]EVSWEEEAEISGSSARYDVALSEQGEFKLLSEEKVPWDQVVMTSLALVGAAIHPFALLLVLAGWLFHV--RGARR  130
Query_10018  1         STNIAGHADSRLDSRGSVWMDTKKATSYLTKAESWALRNPGYALVAAVLGWSLGTSNAQKV-IFTVMILLIAPAY  74
Query_10019  1    [ 57]DVKWSDEAEVTGESVSYHVSLDVRGDPTLTEDSGPGLEKVLLKVGLMAISGIYPVAIPFALGAWFFLE--KRCKR  130
Query_10020  1         SLTVQTHGESTLSNKKGAWMDSTKATRYLVKTESWILRNPGYALVAAVIGWMLGSNTMQRV-VFAVLLLLVAPAY  74
Query_10021  1    [ 58]DISWEGDAEITGSSERVDVRLDDDGNFQLMNDPGAPWKIWMLRMACLAISAYTPWAILPSVVGFWITL--QYTKR  131
Query_10022  1         SVSIPVHAHSDLTGRGHKWLKGDSVKTHLTRVEGWVWKNKFLTAAFCAVVWMVTDSLPTRF-IVITVALCLAPTY  74
Query_10023  1    [ 56]EVEWNPDLVNEGGEVNLKVRQDAMGNLHLTEVEKEERAMALWLLAGLVASAFHWAGILIVLAVWTLFEMLGSGRR  131
Query_10024  1         MYSFVSEETGTLIVNSVLLFLAFVVFLLVTLAILTALRLCAYCCNIVNVSLVKPSFYVYSRVKNL------NSSR  69
Query_10025  1         ----------------ELLPTQRPTQQMKTTKSWLQKIPLQWFKCTVKEGKLQCRI----------------  40
Query_10026  1    [ 11]RNEWGCRCNDSSDPLVVAASIIGILHLILWILDRLFFKCIYRFFEHGLKRGPSTEGVPESMREEYRKEQQSAVDA  86
Query_10027  1         MLPFVQERIGLFIVNFFIFTVVCAITLLVCMAFLTATRLCVQCMTGFNTLLVQPALYLYNTGRSVVVKFQDSKPP  75
Query_10028  1         MYSFVSEETGTLIVNSVLLFLAFVVFLLVTLAILTALRLCAYCCNIVNVSLVKPTVVYVSRVKNL------NSSE  69
Query_10029  1         ----------------ALENLVILNAASLAGTHGLVSFLVFFCFAWYLKGRWVPGAVYAFYGMWPLLLLLALPQ  59
Query_10030  1         ----------------ALEKLVVLHAASAASCNGFLYFVIFFVAAWYIKGRVVPLATYSLTGLWSFGLLLLALPQ  59
Query_10031  1         ----------------ALENLVTLNAVAAAGTHGIGWYLVAFCAAWYVRGKLVPLVTYSLTGLWSLALLVLLLPQ  59
Query_10032  1         ----------------ALSNLININAASAAGAQGFWYAILFICIVWHVKGRFPAAAAYAACGLWPCFLLLLMLPY  59
```

Figure 3. 1: Multiple allignment of viroporin sequences from different virsuses

Alignment Parameters	
Gap penalties	-11,-1
End-Gap penalties	-5,-1

CDD Parameters	
Use RPS BLAST	on
Blast E-value	0.005
Find Conserved columns and Recompute	on

Query Clustering Parameters	
Use query clusters	on
Word Size	4
Max cluster distance	0.8
Alphabet	SE-B15

Figure 3. 2: Alignment parameters

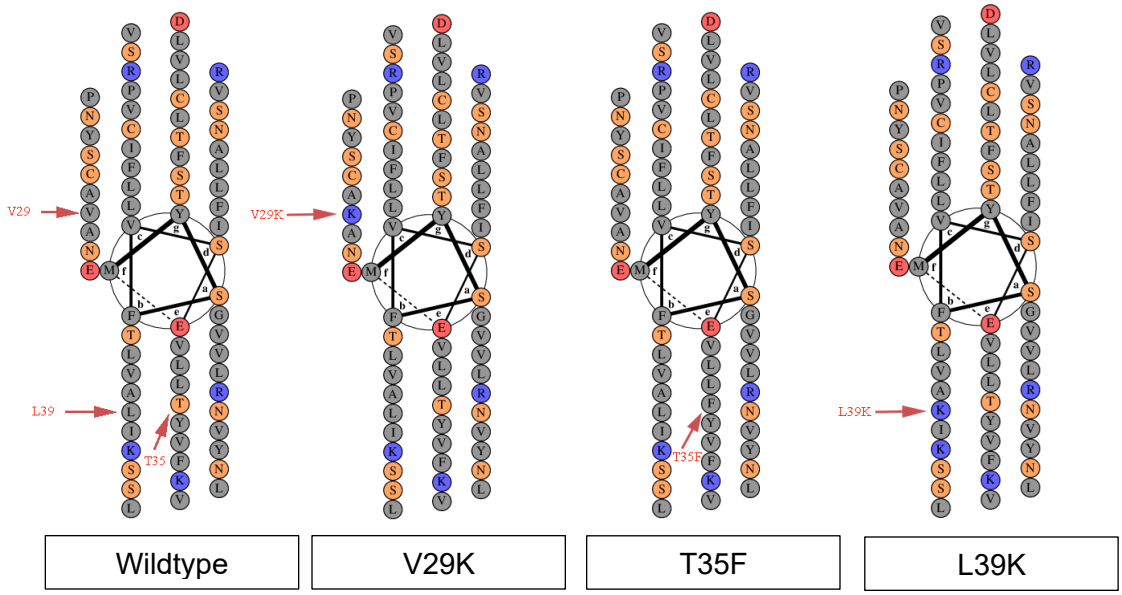

Figure 3. 3: Helical wheels showing the loactions of the mutations on the a-helical structure of the E-protein

```
V29K        MYSFVSEETGTLIVNSVLLFLAFVVFLLKTLAILTALRLCAYCCNIVNVSLVKPSFYVYS    60
T35F        MYSFVSEETGTLIVNSVLLFLAFVVFLLVTLAILFALRLCAYCCNIVNVSLVKPSFYVYS    60
Wildtype    MYSFVSEETGTLIVNSVLLFLAFVVFLLVTLAILTALRLCAYCCNIVNVSLVKPSFYVYS    60
L39K        MYSFVSEETGTLIVNSVLLFLAFVVFLLVTLAILTALRKCAYCCNIVNVSLVKPSFYVYS    60
            ************************** ***** *** ********************

V29K        RVKNLNSSRVPDLLV   75
T35F        RVKNLNSSRVPDLLV   75
Wildtype    RVKNLNSSRVPDLLV   75
L39K        RVKNLNSSRVPDLLV   75
            ***************
```

Figure 3. 4: Amino acid sequences of the Wildtype and mutations

2. *Primer design:*

Based on the results of the bioinformatical search the primers were designed as can be seen in Figure 3.5.

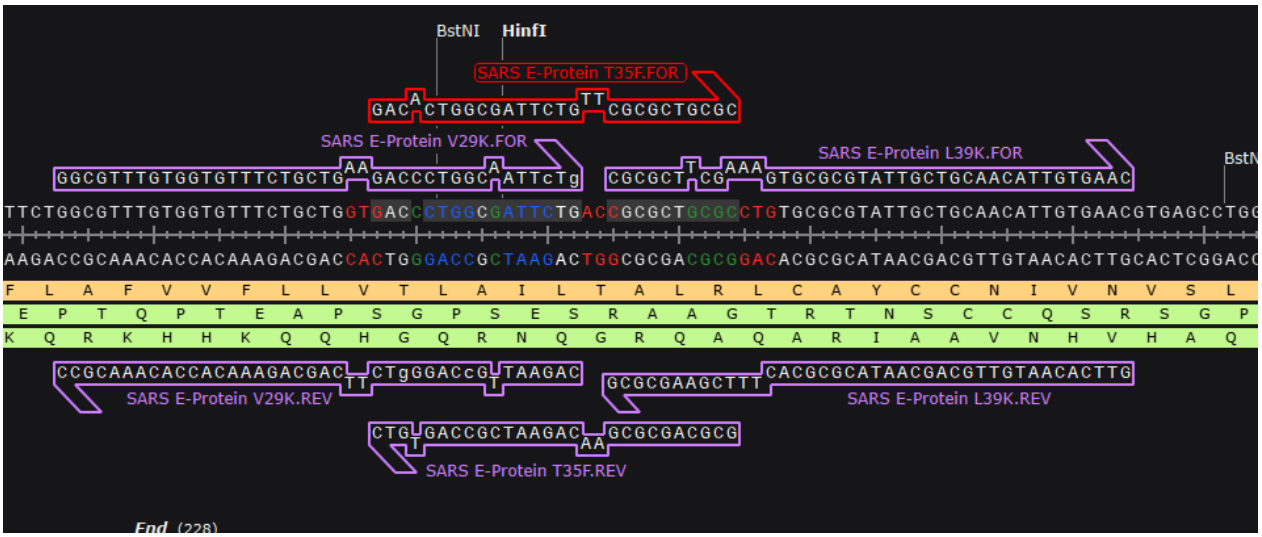

Figure 3. 5: Allignment of the designed primers on the wildtype DNA

3. *Determined DNA concentration using Qubit:*

The obtained DNA concentrations from the minipreparation as well as the midipreparation can be seen in Table 3.1.

Mutation	Miniprep Concentration	Midiprep Concentration
L39K	18.4 ng/µl	2.41 µg/µl
T35F	23.6 ng/µl	2.6 µg/µl
V29K	7.6 ng/µl	1.99 µg/µl

Table 3. 1: Concentrations of the mutated DNA obtained from mini and midi preparations

4. *Test Digest (test for insert):*

The vector transformed in step 10 was tested for the presence or absence of the insert using a test digest described in step 12 of the methodology. The resulting gel of this digest can be seen in Figure 3.6.

Based on the gel in Figure 3.6; samples 1.2 was chosen for mutation L39K, sample 2.2 was chosen for mutation T35F, and sample 3.3 was chosen for mutation V29K.

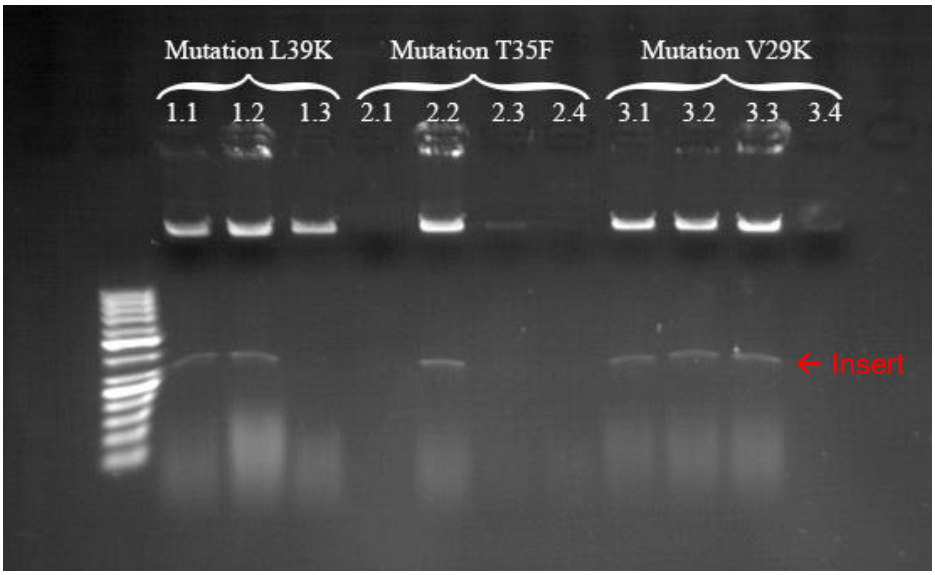

Figure 3. 6: Gel obtained from the digestion products of the three mutations L39K,T35F, and V29K. The desired lanes are the one that contain a band at insert size of ~500 bp

5. *Test Digest (test for mutation):*

The success of the mutagenesis was tested for as described in step 13 of the methodology. A theoretical image of the gel that results from the digestion and electrophoresis is seen in Figure 3.7. While an example of one of the digestions can be seen in Figure 3.8 (this was a failed attempt [failed mutagenesis]).

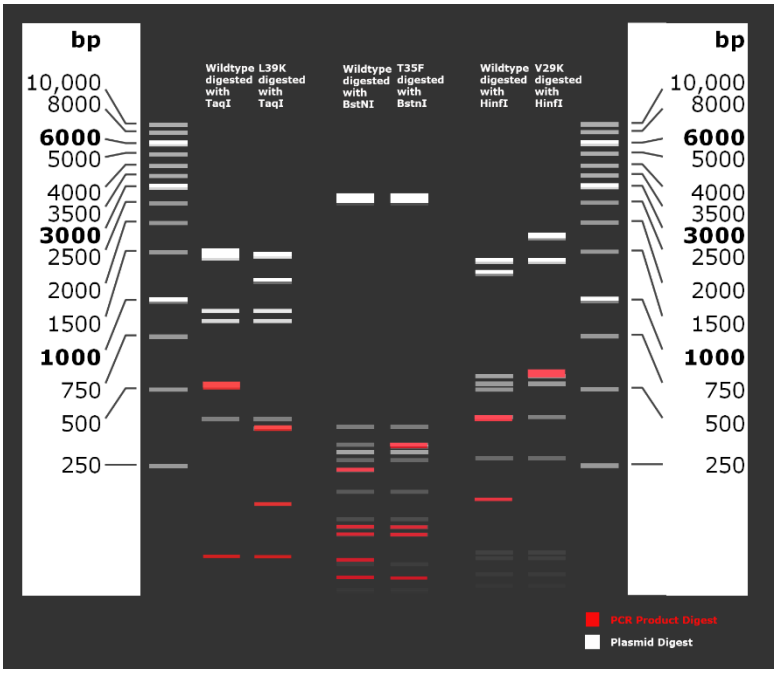

Figure 3. 7: Simulated gel showing the Digestion products of the three mutations with each's respective restriction Enzyme.

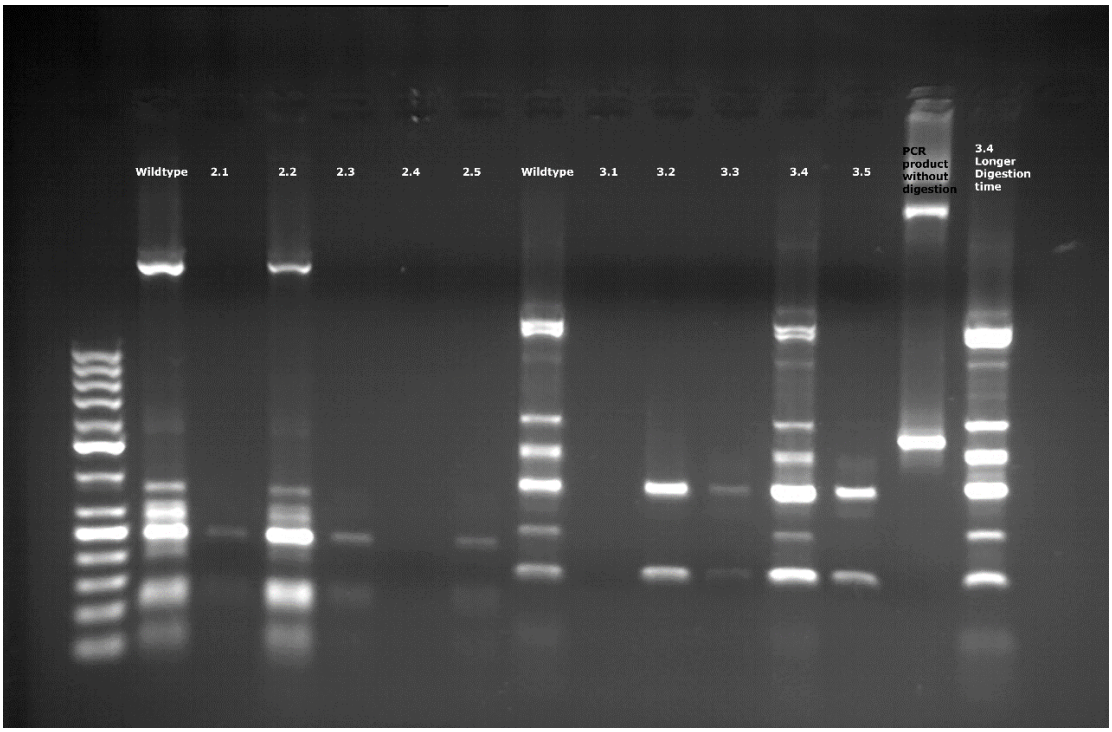

Figure 3. 8: Example Gel run of a test digest for selection of colonies containing the desired mutation. In this image, T35F (label 2.1-2.5) and V29K (Label 3.1-3.5) digestion pattern can be seen and compared to the wildtype digestion pattern

6. *Sequencing:*

The results from the sequencing showed that all 3 mutagenesis reactions were successful. This can be seen in Figure 3.9 to Figure 3.11. Although some other mutations occurred, all of them were outside the ORF. Also, for mutation T35F the silent mutated base responsible for the removal of a BstNI restriction site had mutated back to the wildtype form. This can be seen in Figure 3.10 as the base highlighted in yellow. This was not a big problem however since the purpose of this silent mutation was only to allow testing for the success of the mutagenesis and since the sequencing proved the success of the mutagenesis, the silent mutation became redundant.

Wildtype vs sequenced L39K

Sequence ID: Query_27365 **Length:** 1163 **Number of Matches:** 1

Range 1: 3 to 526 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
933 bits(505)	0.0	518/524(99%)	1/524(0%)	Plus/Plus

```
Query  40   CAACTGCACCTC-GGTTCTATCGATTGAATTCGCCGCCACCATGTGGACCCCGCGAGTGC  98
Sbjct  3    ............G..................A.........................  62

Query  99   CCCCTCCACGCCCGGCCCTGTCGTTTTTCCTGCTGTTGCTCCTGGGGGTCACCTATGGCC  158
Sbjct  63   ............................................................  122

Query  159  TTTTCCCAGAAGAACCACCTCCACTCAGCGTGGCTGAGGAGCAAAAGCTCATTTCTGAAG  218
Sbjct  123  ............................................................  182

Query  219  AGGACTTGTTGCGGAAACGACGAGAACAGTTGAAACACAAACTTGAACAGCTACGGAACT  278
Sbjct  183  ............................................................  242

Query  279  CTTGTGCGGGATCCATGTATAGCTTTGTGAGCGAAGAAACCGGCACCCTGATTGTGAACA  338
Sbjct  243  ............................................................  302

Query  339  GCGTGCTGCTGTTTCTGGCGTTTGTGGTGTTTCTGCTGGTGACCCTGGCGATTCTGACCG  398
Sbjct  303  ............................................................  362

Query  399  CGCTGCGCCTGTGCGCGTATTGCTGCAACATTGTGAACGTGAGCCTGGTGAAACCGAGCT  458
Sbjct  363  ....T..AAA.....................................................  422

Query  459  TCTATGTGTATAGCCGCGTGAAAAACCTGAACAGCAGCCGCGTGCCGGATCTGCTGGTGT  518
Sbjct  423  ............................................................  482

Query  519  GACTGCAGAAGCTTGGCCGCCATGGCCCAACTTGTTTATTGCAG  562
Sbjct  483  ..........................................  526
```

Figure 3. 9: Comparison of the Sequenced L39K mutation DNA to the E-protein Wildtype DNA

Wildtype vs sequenced T35F

Sequence ID: Query_103115 **Length:** 1104 **Number of Matches:** 1

Range 1: 3 to 526 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
950 bits(514)	0.0	521/524(99%)	1/524(0%)	Plus/Plus

```
Query  40   CAACTGCACCTC-GGTTCTATCGATTGAATTCGCCGCCACCATGTGGACCCCGCGAGTGC  98
Sbjct  3    ............G.............................................  62

Query  99   CCCCTCCACGCCCGGCCCTGTCGTTTTTCCTGCTGTTGCTCCTGGGGGTCACCTATGGCC  158
Sbjct  63   ............................................................  122

Query  159  TTTTCCCAGAAGAACCACCTCCACTCAGCGTGGCTGAGGAGCAAAAGCTCATTTCTGAAG  218
Sbjct  123  ............................................................  182

Query  219  AGGACTTGTTGCGGAAACGACGAGAACAGTTGAAACACAAACTTGAACAGCTACGGAACT  278
Sbjct  183  ............................................................  242

Query  279  CTTGTGCGGGATCCATGTATAGCTTTGTGAGCGAAGAAACCGGCACCCTGATTGTGAACA  338
Sbjct  243  ............................................................  302

Query  339  GCGTGCTGCTGTTTCTGGCGTTTGTGGTGTTTCTGCTGGTGACCCTGGCGATTCTGACCG  398
Sbjct  303  ............................................................TT..  362

Query  399  CGCTGCGCCTGTGCGCGTATTGCTGCAACATTGTGAACGTGAGCCTGGTGAAACCGAGCT  458
Sbjct  363  ............................................................  422

Query  459  TCTATGTGTATAGCCGCGTGAAAAACCTGAACAGCAGCCGCGTGCCGGATCTGCTGGTGT  518
Sbjct  423  ............................................................  482

Query  519  GACTGCAGAAGCTTGGCCGCCATGGCCCAACTTGTTTATTGCAG  562
Sbjct  483  ..........................................  526
```

Figure 3. 10: Comparison of the Sequenced T35F mutation DNA to the E-protein Wildtype DNA

Wildtype vs sequenced V29K

Sequence ID: Query_2361 Length: 817 Number of Matches: 1

Range 1: 3 to 526 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
939 bits(508)	0.0	519/524(99%)	1/524(0%)	Plus/Plus

```
Query  40   CAACTGCACCTC-GGTTCTATCGATTGAATTCGCCGCCACCATGTGGACCCCGCGAGTGC  98
Sbjct  3    ...........G..................A.........................  62

Query  99   CCCCTCCACGCCCGGCCCTGTCGTTTTTCCTGCTGTTGCTCCTGGGGGTCACCTATGGCC  158
Sbjct  63   ............................................................  122

Query  159  TTTTCCCAGAAGAACCACCTCCACTCAGCGTGGCTGAGGAGCAAAAGCTCATTTCTGAAG  218
Sbjct  123  ............................................................  182

Query  219  AGGACTTGTTGCGGAAACGACGAGAACAGTTGAAACACAAACTTGAACAGCTACGGAACT  278
Sbjct  183  ............................................................  242

Query  279  CTTGTGCGGGATCCATGTATAGCTTTGTGAGCGAAGAAACCGGCACCCTGATTGTGAACA  338
Sbjct  243  ............................................................  302

Query  339  GCGTGCTGCTGTTTCTGGCGTTTGTGGTGTTTCTGCTGGTGACCCTGGCGATTCTGACCG  398
Sbjct  303  ..........................AA.........A.........  362

Query  399  CGCTGCGCCTGTGCGCGTATTGCTGCAACATTGTGAACGTGAGCCTGGTGAAACCGAGCT  458
Sbjct  363  ............................................................  422

Query  459  TCTATGTGTATAGCCGCGTGAAAAACCTGAACAGCAGCCGCGTGCCGGATCTGCTGGTGT  518
Sbjct  423  ............................................................  482

Query  519  GACTGCAGAAGCTTGGCCGCCATGGCCCAACTTGTTTATTGCAG  562
Sbjct  483  ..........................................  526
```

Figure 3. 11: Comparison of the Sequenced V29K mutation DNA to the E-protein Wildtype DNA

Figure 3. 12: Part of the electropherogram of mutation L39K

7. *Transfection of HEK 293 Cells:*

In the transfection step the GFP and the other plates were investigated under the microscope (Figure 3.13 – Figure 3.17). The fluorescence of the cells in the GFP plate (Figure 3.15) demonstrates that the transfection was successful.

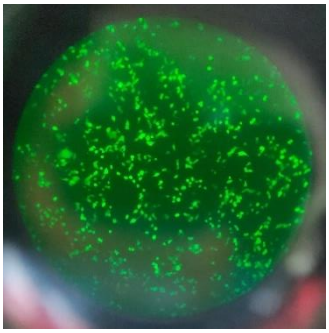

Figure 3. 13: GFP plate

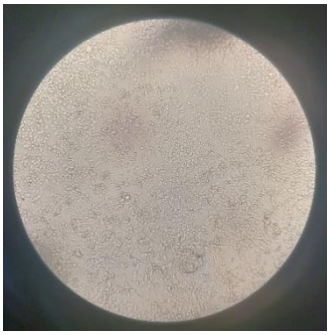

Figure 3. 14: L39K plate

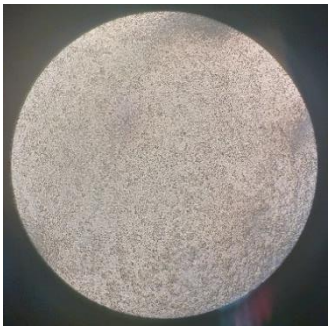

Figure 3. 15: T35F plate

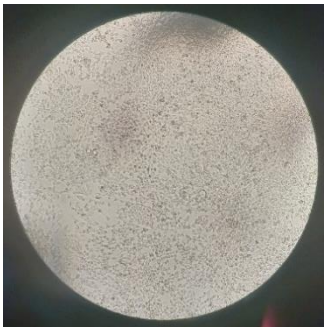

Figure 3. 16: V29K plate

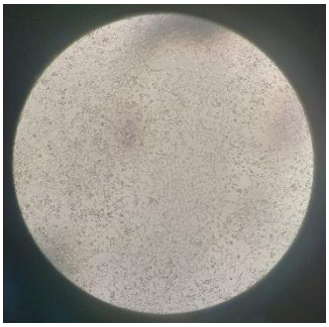

Figure 3. 17: Wildtype E-protein

8. *Western blot (1):*

In this western blot (Figure 3.18) the T35F and the L39K mutated E-proteins bands were visible as thick bands while the 40a wildtype, V29K, and the GFP lanes were empty. This was expected for the GFP lane as it did not contain any myc-tag labelled proteins and therefore should not have had any visible bands in its lane. However, the 40a wildtype and the T35F lanes should not be empty. This result might be due to the highly unstable nature of the E-protein, but if that is the case then why did the V29K and the L39K mutated E-proteins bands show up? A possible explanation to this is that the two mutations stabilized the E-protein and therefore the protein was not degraded while undergoing the steps required for the blot preparation. Further research into this is required to ascertain if this hypothesis is correct or not.

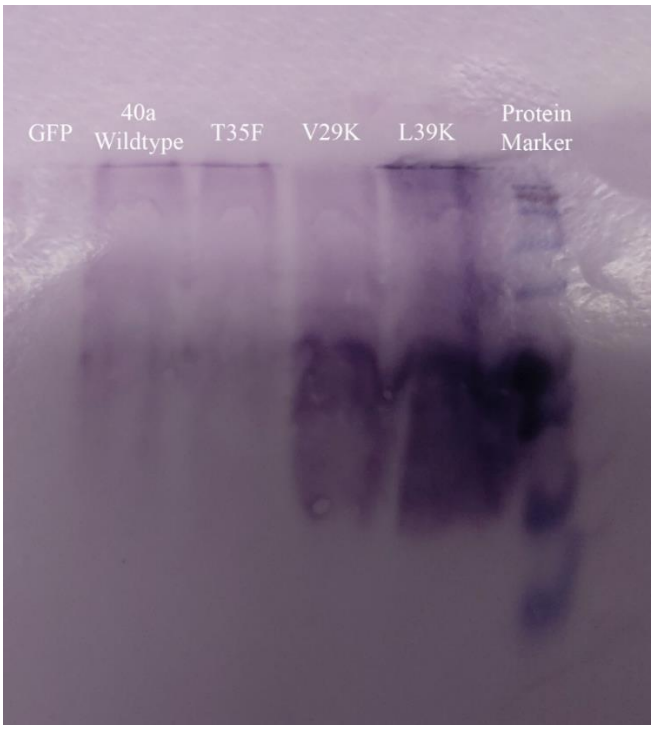

Figure 3. 18: Photo of western blot (1) showing a thick band in the V29K and L39K lanes while no bands in the GFP, E-protein Wildtype, or T35F mutation lanes.

9. *Western blot (2):*

Regarding western blot (2) seen Figure 3.19, all lanes showed bands including the GFP. This is not optimal since as discussed above, theoretically there should be no bands in the GFP lane. What this implies is that the band present in the GFP is unspecific to the E-protein and therefore should be excluded from all the other lanes. Although, since there was only one band in the GFP lane, this is not a big problem. It is worth note to notice that the 40a wildtype and the V29K lanes were not empty as in western blot (1) which suggests that in fact, the second protocol for the membrane preparation (step 18 in methodology) was more suitable to counter the highly unstable nature of the E-protein.

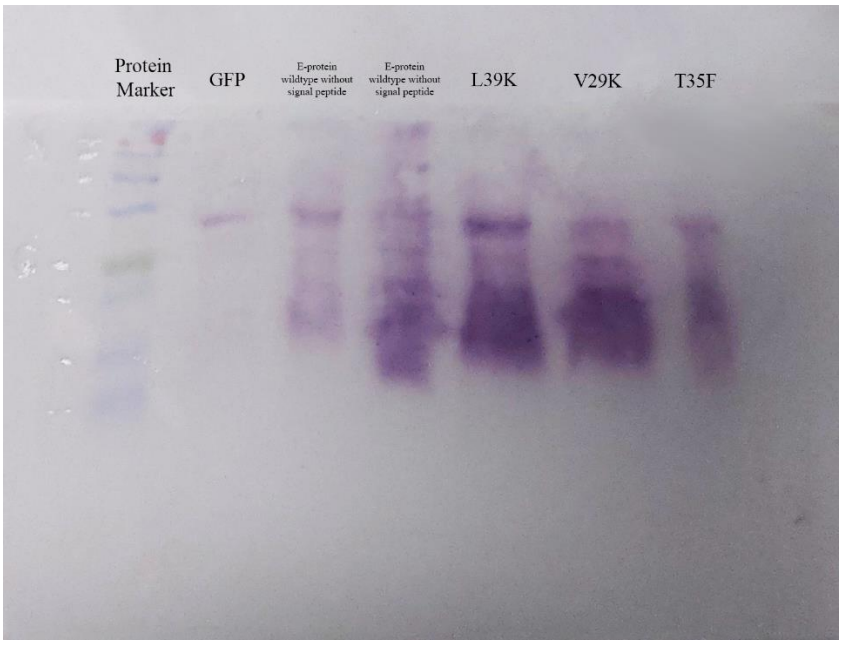

Figure 3. 19: Photo of western blot (2) showing a unspecific singular band in the GFP lane and multimeric bands in remaining wildtype and three mutations lanes. The lowest band is it a size of 60-80 residues which correlates with the size of E-protein of 75 residues

10. MTT:

Figure 3.20 shows that mutation V29K and L39K were less toxic to HEK 293 cells than the 40a wildtype. While mutation T35F was the same toxicity as the E-protein wildtype within standard error. This result suggests that mutation V29K and L39K decreased the activity of the E- protein and therefore it is very likely that valine at position 29 and Leucine at position 39 are part of the functional region of the protein.

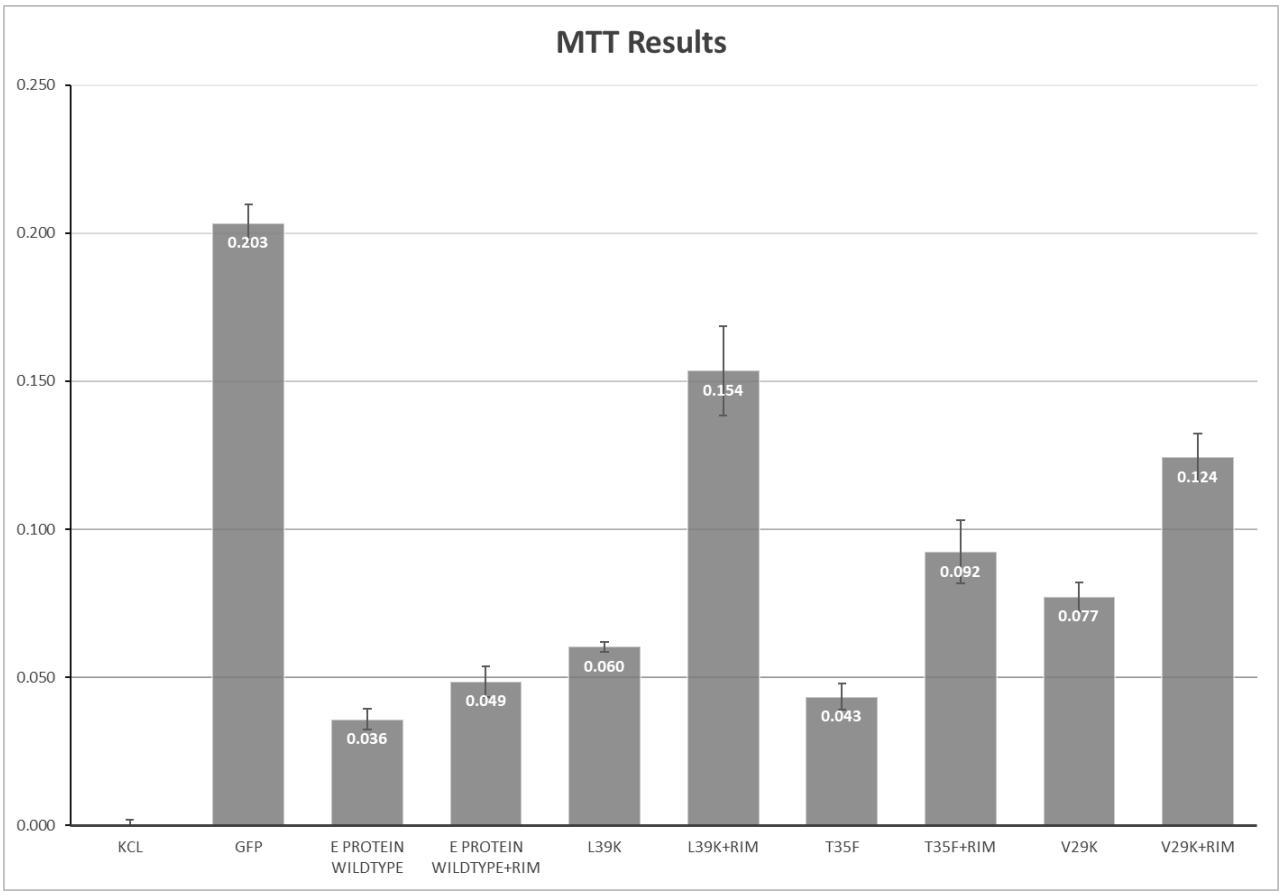

Figure 3. 20: MTT plate results plotted on a bar chart. The y-axis is the measured absorbance and is directly proportional to the number of viable cells

11. Dot blot:

The dot blot (Figure 3.21) results are correlated to what was expected theoretically. The GFP lane showed no/weak dots, and the dot shapes visible in the figure are probable due to the need for more washings of the secondary antibody. The mutations L39K and V29K demonstrated a higher dot colour intensity over the E-protein wildtype and the T35F mutation as can be seen in Figure 3.22. While, T35F had only a slightly higher dot intensity over the E-protein Wildtype. This suggests that either the L39K and V29K are expressed more than the E-protein wildtype and the T35F mutation or that the L39K and V29K are more stable than the wildtype and T35F mutation. The second possibility aligns with the results obtained from western blot (1).

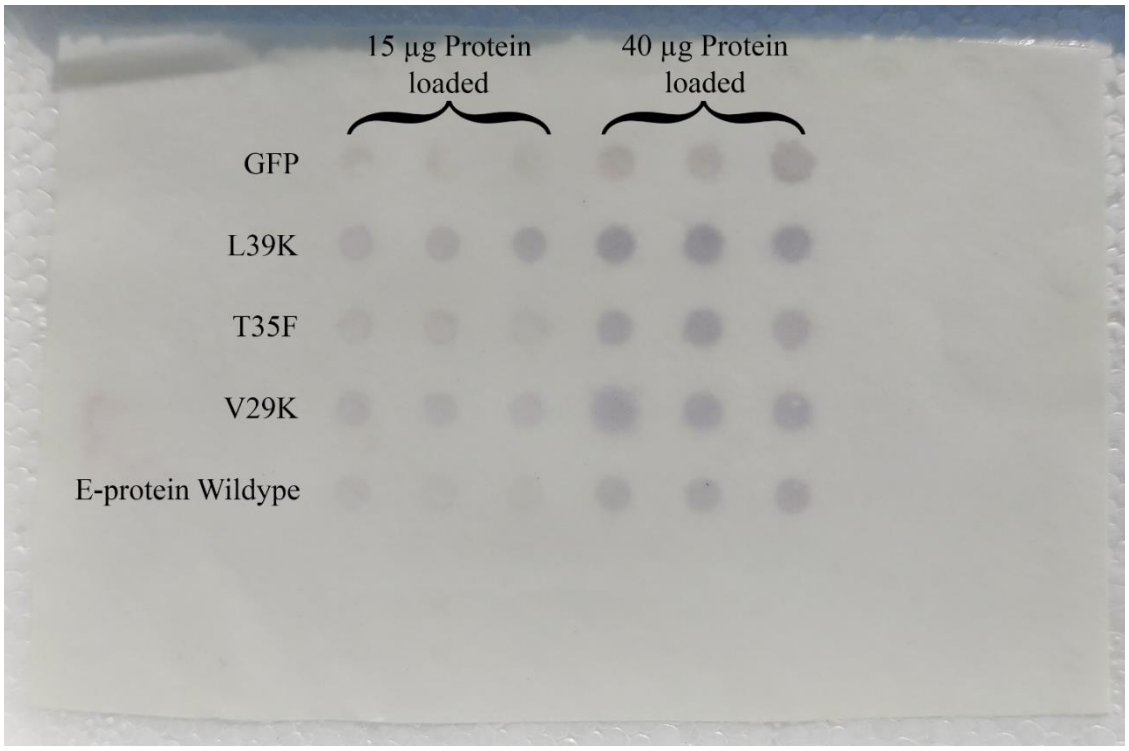

Figure 3. 21: Photo of the developed Dot blot

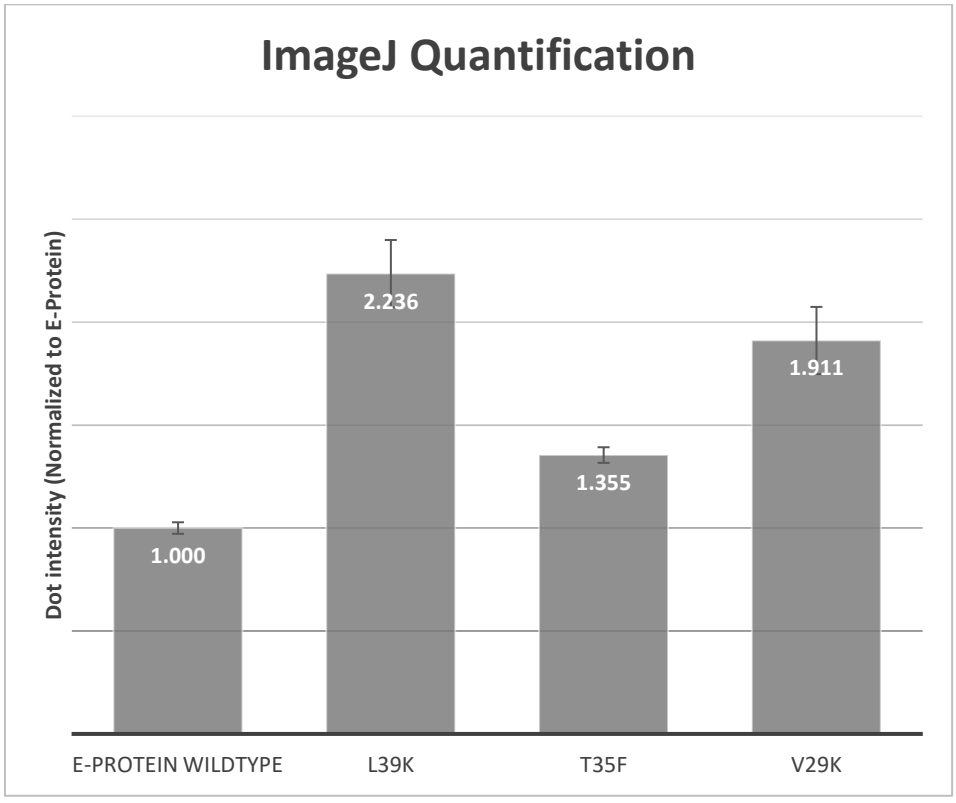

Figure 3. 22: Bar chart of the ImageJ relative quantification, normalized with respect to the E-protein

Mutations L39K and V29K showed the most prominent change in the E-protein characteristics, as the results suggests that these two mutations increase the stability of E-protein while also decreasing its cytotoxic activity (as mentioned before). A plausible explanation to this is that both L39K and V29K mutations cause an exchange of an uncharged amino acid (Leucine and Valine) into the positively charged Lysine. This causes the pore of the E-protein pentamer to become more positive in charge and repel the Ca^{2+} transportee, thus decreasing the activity of the protein. This finding would align with previous research demonstrating the evolutionary tendency of the E-protein of MRSE, SARS-Cov, and SARS-Cov-2 to become more positive in charge, less active, and more stable[14].

It is also worth noting, that these results suggest the possibility of a conserved functional region for multiple viroporins from different viruses. If this is the case, this functional region could be an interesting target for future antiviral drug research.

Conclusion

The E-protein is a viroporin of the SARS-Cov-2 virus that caused the 2019 pandemic. Its activity is vital for virion assembly and pathogenesis of the virus, therefore a better understanding of its structure and functional regions can lead to new drugs and treatments. In this thesis, a possible functional region of the E-protein suggested by bioinformatical methods was investigated by means of mutagenesis. The 3 exchange mutations; L39K, T35F, and V29K were induced into the E-protein and then compared to the Wildtype (unmutated) E-protein in multiple experiments. The experiments conducted were MTT, Western blot, and Dot Blot. The results indicated that mutations L39K and V29K were stabilizing and decreased the cytotoxic activity of the E-protein compared to the wildtype, while T35F did not show any significant difference to the wildtype in either activity or stability. This Suggests that position L39 and V29 are part of the functional region of the E-protein.

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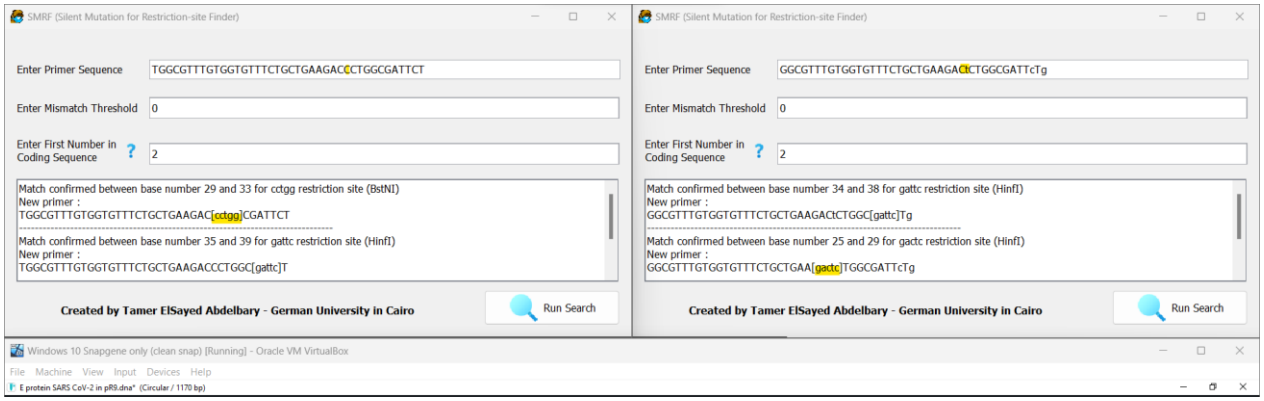
Appendix

Software:



The website to download it from: https://tamer-elsayed.github.io/SMRF/

Layout of the 96 well plate and calculations:

96 well plate

	1	2	3	4	5	6	7	8	9	10	11	12
a	x	GFP	40a	40a	Mut1	Mut1	Mut2	Mut2	Mut3	Mut3	Ctr	x
b	x	#35	3aSP	Rim50		Rim50		Rim50		Rim50	PEI only	x
c	x											x
d	x											x
e	KCl	GFP	40a	40a	Mut1	Mut1	Mut2	Mut2	Mut3	Mut3	Ctr	x
f	KCl	#35	3aSP	Rim50		Rim50		Rim50		Rim50	PEI only	x
g	KCl											x
h	KCl											x

Tube 1					
	No. Wells	**EMEM 7.5 ul (excess to 8)**	**My DNA Concentration**	**DNA 0.3 ug (exess to 0.32) ul**	**PEI (ul)**
GFP	8	64	1	2.56	4.8
40a	8	64	1	2.56	4.8
40a Rim	8	64	1	2.56	4.8
Mut1	8	64	1.62	1.580247	4.8
Mut1 rim	8	64	1.62	1.580247	4.8
Mut2	8	64	0.965	2.65285	4.8

Tube 2		
	EMEM	**PEI**
PEI stock	588.8	51.2

Mut2 rim	8	64	0.965	2.65285	4.8
Mut3	8	64	0.793	3.228247	4.8
Mut3 rim	8	64	0.793	3.228247	4.8
PEI only	8	64	1	2.56	4.8
final	80	640		25.16269	51.2

	con 30x uM	No. Wells	stock Inh. uM	Vol inh 30x needed	vol inh stock	volume to add
RIM50	1500	32	2000	160	120	40