Introduction

Site-directed mutagenesis is a PCR-based, molecular biology method used to insert specific mutations in a known DNA sequence. It is commonly done to study the structure and function of DNA, RNA, and proteins. It can also be used to study protein-protein interactions, binding domains of the protein, or active sites of enzymes. In this method, we use synthetic oligonucleotides to experimentally alter a nucleotide sequence of our interest. Most commonly a double-stranded DNA sequence is used as a template. Using single-stranded DNA for the template can be labor-intensive and time-consuming [1]. In this technique, we design an oligonucleotide primer that is complementary to a part of our sequence of interest, only with a slight mismatch, which is the mutation we want to add to the sequence. Along with point mutations, this technique can also be used to insert multiple mutations, insertions, or deletions [2]. The mutation in the primer is such that the mutation is present in the center surrounded by a perfectly complementary sequence to the template, hence the primer binds with the template. The primers should be of appropriate length so they can form a bonding with the template DNA and form a stable DNA duplex. This step is known as primer annealing. This is followed by DNA synthesis so the primer can grow and form a complete DNA strand along the lines of the template. For this step, DNA polymerase along with dNTPs are added to the reaction. This step is known as the elongation. Elongation is followed by DNA amplification in which multiple copies of the mutant DNA duplex are formed. Now our reaction mixtures contain both template DNA and mutant DNA. However, we require only mutant DNA for our further studies.

To filter out only the mutant DNA, the PCR product is digested with endonuclease Dpn, which digests the methylated sequences of the template DNA. This leaves behind only mutated DNA in the reaction mixture, as they are unmethylated. Now our mutated sequence of interest is ready to be transformed into host cells.

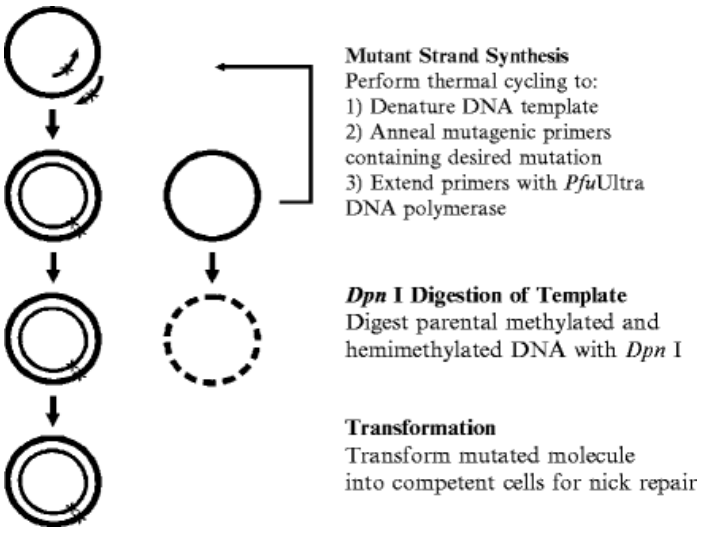

Figure1: The above figure is a brief overview of site-directed mutagenesis [1].

Thrombin-activable fibrinolysis inhibitor (TAFI), is an enzyme that is encoded by the gene CPB2 in humans. It was discovered in 1989 and initially given the name carboxypeptidase U. It was later given many other names but is commonly called TAFI. It has a molecular weight of 55 kDa and is synthesized in the liver. It is found circulating in the blood at a concentration of 70-275 nmol/L. The TAFI gene contains 11 exons and spans across 48 kb of genomic DNA. It is present on chromosome 13.

TAFI plays an active role in haemostasis, which is the formation of blood clots at the places of cuts in our body. Plasma fibrinogen is converted to fibrin in the presence of thrombin, which leads to the formation of an insoluble fibrin clot. Within the fibrin clot, there is continuous fibrin formation and fibrinolysis. The clot formation and stability of the clot are overlooked by thrombin. Not only that, but thrombin is also responsible for activating TAFI which strengthens the fibrin clot and protects it from lysis. Therefore, any defects in TAFI activation could lead to a range of bleeding disorders. Whereas an increase in thrombin generation which in turn increase TAFI activation might lead to thrombotic disorders [3].

Materials and Methods

1. The first step of the experiment is to do restriction digestion of TAFI expressed plasmid. This plasmid will be our plasmid DNA for restriction digestion and template for PCR-based site-directed mutagenesis. The materials required for the first step are as follows in the given quantities.

Component		**Control**	**Reaction 1**	**Reaction 2**	**Reaction 3**
		Volume (µl)			
1	H_2O	13	12.5	12.5	12
2	10× CutSmart buffer	2	2	2	2
3	Plasmid DNA * (200 ng/µl)	5	5	5	5
4	AvrII	0	0.5	0	0.5
5	SpeI	0	0	0.5	0.5
	Total volume	*20*	*20*	*20*	*20*

The following components are to be added in a tube and mixed properly. Incubate the tube in a water bath for 1 hour and conclude by adding loading dye.

2. The second step is to perform site-directed mutagenesis. The following reagents are to be added in a tube that already contains dNTP:

   7.5 µl H_2O

   5 µl 5× Phusion HF reaction buffer

   2 µl template plasmid (TAFI-pcDNA4a; 10

   ng/µl) 5 µl sense primer (25 ng/µl)

   5 µl antisense primer (25 ng/µl)

   0.5 µl Phusion high fidelity DNA

   polymerase Total volume = 25 µl

   After adding the reagents and mixing, the tube is kept in a thermal cycler. The following steps undergo in the thermal cycler: Denaturation, annealing, and elongation.

3. The next step of the reaction is to perform DpnI digestion and bacterial transformation of site-directed mutagenesis reaction. We start by adding the DpnI restriction enzyme to the reaction tube. Next, we add a portion of the reaction mixture to another tube containing bacterial cells. LB broth is added to the bacterial cells and the tube is left for transformation to take place. The bacterial cells are then plated on an LB-agar plate containing ampicillin.

4. The fourth step is to perform agarose gel electrophoresis. A pre-made 1% agarose gel is obtained and the apparatus is set up. The lanes in the gel are loaded in the following manner:

   Lane 1: 1 kb ladder

Lane 2: Control reaction

Lane 3: Reaction 1

Lane 4: Reaction 2

Lane 5: Reaction 3

Lane 6 – 8: Empty

Run the gel on a voltage of 80V for approximately 45 minutes. Examine the gel and take an image.

5. The next step is to remove plasmid DNA from the bacteria that are transformed. Once the plasmid DNA is eluted, we move to the next step.
6. In this step, we measure the concentration of plasmid DNA extracted.

7. The next step is the restriction analysis of clones from site-directed mutagenesis. To conclude, we retrieve an agarose gel and load the following samples in the given quantities:

    a. 1 kb ladder (10 µl)
    b. Positive wild-type control digest (load entire sample) *given to you*
    c. Positive clone control digest (load entire sample) * given to you * - (has mutation)
    d. Your experimental control digest (24 µl)
    e. Digest of clone 1 (24 µl)
    f. Digest of clone 2 (24 µl)
    g. 6-8. Empty

Run the gel on 80V for 45 minutes. Examine and take an image of the gel.

DNA Translation

The DNA sequence can be translated by using the Expasy website. Follow the following steps:

1. Go to https://web.expasy.org/translate/
2. Select the option “Includes nucleotide sequence
3. Enter the nucleotide sequence in the search box
4. Press translate

Phosphorylation Sites

Phosphote.org can be used to determine the phosphorylation sites of a given DNA segment.

Primer Designing

This can be performed by following the below steps:

1. Go to the website: http://www.bioinformatics.org/primerx/
2. Click on the link: 'enter a mutation in your template DNA sequence'
3. Copy and paste the DNA sequence below in FASTA format into the text area in step 1.
4. Enter the desired mutation
5. Select the appropriate set of primers

Results

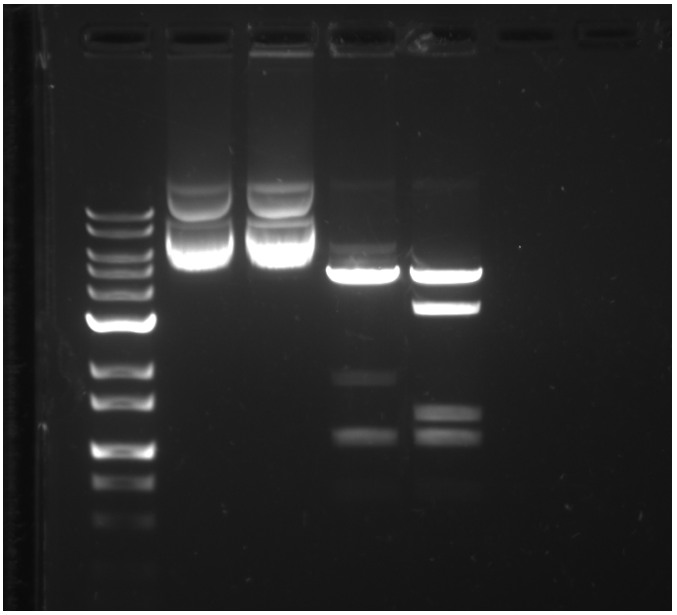

Image 1: The above image shows the gel electrophoresis image from Day2. The components in each lane are as follows from left to right: Lane 1-1 kb ladder, Lane 2-Control reaction, Lane 3- Reaction 1, Lane 4-Reaction 2 and Lane 5- Reaction 3.

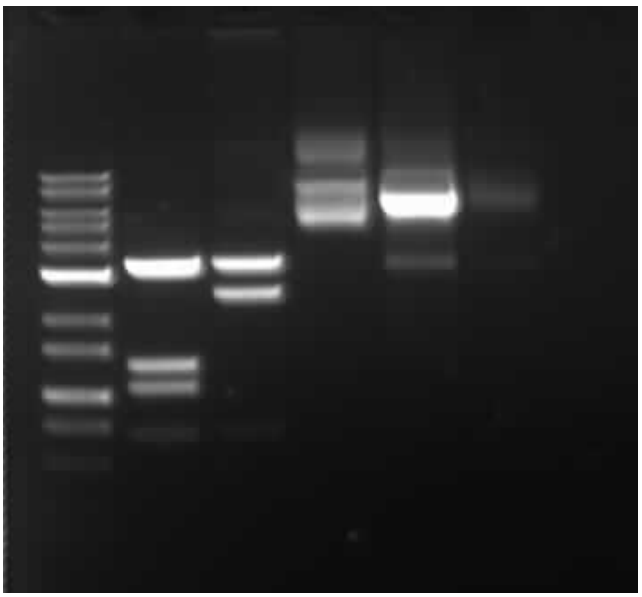

Image2: The above image shows the results from gel electrophoresis performed on day 4. The components in each lane are as follows from left to right: Lane 1-1 kb ladder, Lane 2-Positive control reaction, Lane 3- Reaction 1, Lane 4-Reaction 2 and Lane 5- Reaction 3.

Phosphorylation sites
Thr383
Tyr380
Lys346
Ser338

Table1: The above table shows the list of phosphorylation sites in TAFI.

Primer designing:

The given primers can be used to mutate the DNA sequence such that Serine is converted to Asparagine.

Forward: 5' CTTCGCGTTTCAGAATGGCCAAGTTCTAGC 3'
Reverse: 5' GCTAGAACTTGGCCATTCTGAAACGCGAAG 3'

Forward: 5' GTCTTCGCGTTTCAGAATGGCCAAGTTCTAGC 3'
Reverse: 5' GCTAGAACTTGGCCATTCTGAAACGCGAAGAC 3'

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You submitted the following DNA sequence:
                .         .         .         .         .      1   ATGAAGCTTTGCAGCCTTGCAGTCCTTGTACCCATTGTTCTCTTCTGTGA   50
    51   GCAGCATGTCTTCGCGTTTCAGAGTGGCCAAGTTCTAGCTGCTCTTCCTA   100    101   GAACCTCTAGGCAAGTTCAAGTTCTACAGAATCTTACTACAACATATGAG   150
   151   ATTGTTCTCTGGCAGCCGGTAACAGCTGACCTTATTGTGAAGAAAAAACA   200    201   AGTCCATTTTTTTTGTAAATGCATCTGATGTCGACAATGTGAAAGCCCATT   250
   251   TAAATGTGAGCGGAATTCCATGCAGTGTCTTGCTGGCAGACGTGGAAGAT   300    301   CTTATTCAACAGCAGATTTCCAACGACACAGTCAGCCCCCGAGCCTCCGC   350
   351   ATCGTACTATGAACAGTATCACTCACTAAATGAAATCTATTCTTGGATAG   400    401   AATTTATAACTGAGAGGCATCCTGATATGCTTACAAAAATCCACATTGGA   450
   451   TCCTCATTTGAGAAGTACCCACTCTATGTTTTAAAGGTTTCTGGAAAAGA   500    501   ACAAACAGCCAAAAATGCCATATGGATTGACTGTGGAATCCATGCCAGAG   550
   551   AATGGATCTCTCCTGCTTTCTGCTTGTGGTTCATAGGCCATATAACTCAA   600    601   TTCTATGGGATAATAGGGCAATATACCAATCTCCTGAGGCTTGTGGATTT   650
   651   CTATGTTATGCCGGTGGTTAATGTGGACGGTTATGACTACTCATGGAAAA   700    701   AGAATCGAATGTGGAGAAAGAACCGTTCTTTCTATGCGAACAATCATTGC   750
   751   ATCGGAACAGACCTGAATAGGAACTTTGCTTCCAAACACTGGTGTGAGGA   800    801   AGGTGCATCCAGTTCCTCATGCTCGGAAACCTACTGTGGACTTTATCCTG   850
   851   AGTCAGAACCAGAAGTGAAGGCAGTGGCTAGTTTCTTGAGAAGAAATATC   900    901   AACCAGATTAAAGCATACATCAGCATGCATTCATACTCCCAGCATATAGT   950
   951   GTTTCCATATTCCTATACACGAAGTAAAAGCAAAGACCATGAGGAACTGT   1000   1001   CTCTAGTAGCCAGTGAAGCAGTTCGTGCTATTGAGAAAACTAGTAAAAAT   1050
  1051   ACCAGGTATACACATGGCCATGGCTCAGAAACCTTATACCTAGCTCCTGG   1100   1101   AGGTGGGGACGATTGGATCTATGATTTGGGCATCAAATATTCGTTTACAA   1150
  1151   TTGAACTTCGAGATACGGGCACATACGGATTCTTGCTGCCGGAGCGTTAC   1200   1201   ATCAAACCCACCTGTAGAGAAGCTTTTGCCGCTGTCTCTAAAATAGCTTG   1250
  1251   GCATGTCATTAGGAATGTTACCGGT                            1275

Which translates into the following protein sequence:
                .         .         .         .         .      1   MKLCSLAVLVPIVLFCEQHVFAFQSGQVLAALPRTSRQVQVLQNLTTTYE   50
    51   IVLWQPVTADLIVKKKQVHFFVNASDVDNVKAHLNVSGIPCSVLLADVED   100    101   LIQQQISNDTVSPRASASYYEQYHSLNEIYSWIEFITERHPDMLTKIHIG   150
   151   SSFEKYPLYVLKVSGKEQTAKNAIWIDCGIHAREWISPAFCLWFIGHITQ   200    201   FYGIIGQYTNLLRLVDFYVMPVVNVDGYDYSWKKNRMWRKNRSFYANNHC   250
   251   IGTDLNRNFASKHWCEEGASSSSCSETYCGLYPESEPEVKAVASFLRRNI   300    301   NQIKAYISMHSYSQHIVFPYSYTRSKSKDHEELSLVASEAVRAIEKTSKN   350
   351   TRYTHGHGSETLYLAPGGGDDWIYDLGIKYSFTIELRDTGTYGFLLPERY   400    401   IKPTCREAFAAVSKIAWHVIRNVTG                            425

You then entered the following mutated protein sequence:
                .         .         .         .         .      1   MKLCSLAVLVPIVLFCEQHVFAFQNGQVLAALPRTSRQVQVLQNLTTTYE   50
    51   IVLWQPVTADLIVKKKQVHFFVNASDVDNVKAHLNVSGIPCSVLLADVED   100    101   LIQQQISNDTVSPRASASYYEQYHSLNEIYSWIEFITERHPDMLTKIHIG   150
   151   SSFEKYPLYVLKVSGKEQTAKNAIWIDCGIHAREWISPAFCLWFIGHITQ   200    201   FYGIIGQYTNLLRLVDFYVMPVVNVDGYDYSWKKNRMWRKNRSFYANNHC   250
   251   IGTDLNRNFASKHWCEEGASSSSCSETYCGLYPESEPEVKAVASFLRRNI   300    301   NQIKAYISMHSYSQHIVFPYSYTRSKSKDHEELSLVASEAVRAIEKTSKN   350
   351   TRYTHGHGSETLYLAPGGGDDWIYDLGIKYSFTIELRDTGTYGFLLPERY   400    401   IKPTCREAFAAVSKIAWHVIRNVTG                            425
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Image2: The above image shows the nucleotide sequence of TAFI, the protein sequence and the mutated sequence.

Discussion

Through our experiments, we have been successful in performing site directed mutagenesis and observing the resultant DNA on gel electrophoresis. We have identified the phosphorylation sites of TAFI, and we have designed primers to inculcate mutations of our choice into the DNA to yield a particular product.

By designing the primers to induce site-directed mutagenesis in the TAFI protein, we can meddle with the phosphorylation sites. Since both over-stimulation and under stimulation of TAFI can cause diseases in humans, the property to meddle with the phosphorylation sites can further help us to regulate TAFI.

Further experiments need to be conducted in order to fully understand the effects of site-directed mutagenesis in TAFI protein in humans. More research can be performed to produce protein out of the mutated DNA, so we can understand in depth if this technique holds any promise in the real world.

References:

1. Carrigan P.E., Ballar P., Tuzmen S. (2011) Site-Directed Mutagenesis. In: DiStefano J. (eds) Disease Gene Identification. Methods in Molecular Biology (Methods and Protocols), vol 700. Humana Press, Totowa, NJ.
2. Bachman, J., 2013. Site-directed mutagenesis. *Methods Enzymol*, *529*, 241-248.
3. Bouma, B. N., & Mosnier, L. O. (2006). Thrombin activatable fibrinolysis inhibitor (TAFI)—how does thrombin regulate fibrinolysis?. *Annals of medicine*, *38*(6), 378-388.