**Recombinant DNA Technology**

## Week #1

### Educational Objectives:

By the completion of this lab you should be able to:

1. Demonstrate the ability to isolate and prepare restriction digest samples
2. Understand and explain the principals of the use of restriction endonucleases, site- directed mutagenesis, and bacterial transformation

### Background:

The ability of researchers to manipulate DNA at the molecular level is known as recombinant DNA technology or “genetic engineering”. This technology has revolutionized medicine and biomedical research, biology, environmental science, forensic science, and many

other fields. It has also given birth to an entirely new industry: biotechnology. The term “recombinant DNA” refers to the ability to prepare novel DNA species by “recombining” DNA from different sources. The molecular biology era was launched with the solving of the structure of the DNA double helix by Watson and Crick in the 1950’s. This achievement demonstrated the mechanism by which genetic information could be transmitted from one generation to the next. Subsequent studies led to characterization of the genetic code and elucidation of how genetic information encodes proteins by the transcription of specific genes into messenger RNA (mRNA) and the translation of mRNA into proteins.

In the 1970’s, it became possible to isolate and characterize genes: specific segments of DNA encoding proteins. Interestingly, much of this work was performed using the simplest of organisms: bacteria and the viruses that infect them, known as bacteriophages. In concert with this work, important genetic studies were conducted in yeast (the simplest eukaryote), the plant *A. thalania*, the nematode *C. elegans*, and the fruit fly *D. melanogaster* that led to the identification of many important gene families. Indeed, most of the tools of recombinant DNA technology have arisen from bacteriophage and bacteria. Plasmids are small, circular double-stranded DNA molecules that are common in bacteria and which have been adapted to become key vectors for recombinant DNA. Bacterial species have provided an enormous library of restriction endonucleases, enzymes that cleave double-stranded DNA at specific sites (recognition sequences). Bacteria use these enzymes as a defense mechanism, where they cleave, or ‘restrict’, foreign DNA. In recombinant DNA technology, restriction endonucleases are used as molecular scissors to cleave DNA molecules to allow them to be recombined with other DNA molecules. Bacteriophages have provided researchers with a suite of enzymes that are the workhorses of molecular biology. Amongst these enzymes are DNA and RNA polymerases that synthesize nucleic acids in a template-dependent manner, and DNA ligases that catalyze the formation of phosphodiester bonds to allow assembly of recombinant DNA constructs.

Plasmids and bacteriophage have also been used to construct “libraries” of genomic DNA or cDNA (double-stranded DNA copies of RNA) that allowed for the isolation and characterization of specific genes. These efforts ultimately led to the complete sequencing of the human genome, as well as those of many other organisms. Although protein sequencing was actually developed

before DNA sequencing, the latter technique quickly eclipsed the former as it offers much higher throughput and does not require the prior isolation of high-quality protein, which is difficult in many cases. As the sequences of more and more genes – and thus proteins – were characterized it became possible to use the sequence information to gain insights into the function and evolution of proteins as well as their roles in development, physiology, and disease. In addition, it became possible and desirable to express recombinant versions of proteins, encoded by cloned cDNA’s contained within expression plasmids. Many types of cells have been used to express recombinant proteins, including bacteria, yeast, baculovirus-infected insect cells and cultured mammalian cell lines. Using this technology, it is possible to produce large amounts of proteins and to purify them rigorously to homogeneity, a task that would have been impossible with many low-abundance or unstable natural proteins. For instance, many of not most proteins currently used as drugs are produced as recombinant proteins, including insulin, tissue-type plasminogen activator and Herceptin.

Expression of proteins using recombinant DNA technology also affords the ability to selectively mutate one or more residues in the protein for the purposes of structure/function analyses of the protein. For this purpose, a technique known as site-directed mutagenesis is performed to introduce the desired mutations into the cloned cDNA and is used to alter the nucleotide sequence of cloned DNA at a predefined position or site. This method can be used to change only a single or multiple base pairs, or can be used to create more extensive sequence changes such as deletions and insertions or removal or introduction or restriction enzyme sites.

There are two general methods for oligonucleotide site-directed mutagenesis: Synthetic DNA cassettes and Enzymatic extensions of a mutagenic oligonucleotide. The end result of both methods is similar, an oligonucleotide encoding the desired ‘new’ DNA sequence is inserted into a cloned DNA. The method we will be using in lab is enzymatic extension or more commonly referred to as site-directed mutagenesis (SDM) (Fig 1). SDM is an *in vitro* procedure that utilizes custom designed oligonucleotide primers to confer a desired mutation, insertion or deletion in a double-stranded DNA (dsDNA) plasmid. In this method, a short oligonucleotide generally 20 – 40 nucleotides in length containing the substitution is hybridized to its complementary sequence in a circular manner in the wild-type DNA template. The mutation is introduced to the middle of the sequence and is flanked by a perfect complementary wild-type

sequence so that it’s able to anneal to the template, regardless of the mismatch. The wild-type sequences need to be long enough so a stable DNA duplex can form between the oligonucleotide primer and the template DNA. Following primer annealing, DNA polymerase along with dNTPs are added in order to synthesize the remainder of the complementary strand using the mutagenic oligonucleotide as a primer for DNA strand synthesis. Following PCR amplification, there will be a mixture of both wild-type and mutant DNA. The unique feature of utilizing this system is that its capable to remove the template (parental) DNA (the non-mutated supercoiled dsDNA) prior to bacterial transformation. To accomplish this, the PCR product is digested with the endonuclease Dpn1, which digests methylated sequences in the template DNA. Since the mutated PCR products are unmethylated, following Dpn1 digestion only the newly created mutated DNA is left. The sequence of interest is then transformed into competent cells which are able to perform nick repair and methylate the DNA. The mutation is identified by either DNA sequencing or hybridization. Sequencing uses only one primer, while PCR utilizes two. If we tried to sequence with two different primers, we would get the two sequences back, superimposed on each other and completely unreadable.

Wild-type plasmid vector

(Template DNA)

Oligonucleotides with mutation (Sense and Antisense primers)

Reaction buffer dNTPs

DNA polymerase

Denaturation

Primer annealing

Elongation

PCR products

 Repeat PCR cycles

Mixture of original and modified plasmid DNA

Digest with Dpn1

Only PCR amplified

plasmid DNA remains

 Transform *E.coli*; Select colonies

Prepare plasmid DNA and identify mutation by screening and sequencing

Figure 1: The template DNA (plasmid vector) is first denatured to become single stranded. The sequence of the mutagenic primers is perfectly complementary to the wild-type sequence of the template, except for the mismatched nucleotide(s) that contain the mutant DNA sequence. This oligonucleotide is annealed to the single stranded DNA. DNA polymerase uses this mutagenic oligonucleotide primer to synthesize the complementary strand of DNA containing the newly introduced mutation.

In this laboratory, you will execute several key procedures of recombinant DNA technology, in the context of a mammalian expression plasmid for a protein known as TAFI (Thrombin-activatable fibrinolysis inhibitor), including site-directed mutagenesis of Threonine at position 325 to Isoleucine (T325I), and mapping of the plasmid using restriction enzyme digestion and agarose gel electrophoresis.



Figure 2: Plasmid vector map of the pcDNA.4A vector in which TAFI was cloned into the *Age*I and *Pst*I restriction sites within the multiple cloning site of the vector.

WT.TAFI DNA sequence in FASTA format:

ATGAAGCTTTGCAGCCTTGCAGTCCTTGTACCCATTGTTCTCTTCTGTGAGCAGCATGTCT TCGCGTTTCAGAGTGGCCAAGTTCTAGCTGCTCTTCCTAGAACCTCTAGGCAAGTTCAAG TTCTACAGAATCTTACTACAACATATGAGATTGTTCTCTGGCAGCCGGTAACAGCTGACC TTATTGTGAAGAAAAAACAAGTCCATTTTTTTGTAAATGCATCTGATGTCGACAATGTGA AAGCCCATTTAAATGTGAGCGGAATTCCATGCAGTGTCTTGCTGGCAGACGTGGAAGATC TTATTCAACAGCAGATTTCCAACGACACAGTCAGCCCCCGAGCCTCCGCATCGTACTATG AACAGTATCACTCACTAAATGAAATCTATTCTTGGATAGAATTTATAACTGAGAGGCATC CTGATATGCTTACAAAAATCCACATTGGATCCTCATTTGAGAAGTACCCACTCTATGTTT TAAAGGTTTCTGGAAAAGAACAAACAGCCAAAAATGCCATATGGATTGACTGTGGAATCC ATGCCAGAGAATGGATCTCTCCTGCTTTCTGCTTGTGGTTCATAGGCCATATAACTCAAT TCTATGGGATAATAGGGCAATATACCAATCTCCTGAGGCTTGTGGATTTCTATGTTATGC CGGTGGTTAATGTGGACGGTTATGACTACTCATGGAAAAAGAATCGAATGTGGAGAAAGA ACCGTTCTTTCTATGCGAACAATCATTGCATCGGAACAGACCTGAATAGGAACTTTGCTT CCAAACACTGGTGTGAGGAAGGTGCATCCAGTTCCTCATGCTCGGAAACCTACTGTGGAC TTTATCCTGAGTCAGAACCAGAAGTGAAGGCAGTGGCTAGTTTCTTGAGAAGAAATATCA ACCAGATTAAAGCATACATCAGCATGCATTCATACTCCCAGCATATAGTGTTTCCATATT CCTATACACGAAGTAAAAGCAAAGACCATGAGGAACTGTCTCTAGTAGCCAGTGAAGCAG TTCGTGCTATTGAGAAAACTAGTAAAAATACCAGGTATACACATGGCCATGGCTCAGAAA CCTTATACCTAGCTCCTGGAGGTGGGGACGATTGGATCTATGATTTGGGCATCAAATATT CGTTTACAATTGAACTTCGAGATACGGGCACATACGGATTCTTGCTGCCGGAGCGTTACA TCAAACCCACCTGTAGAGAAGCTTTTGCCGCTGTCTCTAAAATAGCTTGGCATGTCATTA GGAATGTTACCGGT

Translated protein sequence for WT.TAFI

The start codon ATG and stop codon TGA are labeled along with specific restriction enzyme recognition sequences within the WT.TAFI DNA, the 6x-His tag which is part of the vector in which TAFI has been cloned in to (not part of TAFI itself), and the color coded mutations which you will make are highlighted

Start (1)

TAAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGAATTCTGCAGGTTGGGA

2 TGAAGCTTTGCAGCCTTGCAGTCCTTGTACCCATTGTTCTCTTCTGTGAGCAGCATGTCT

1 M K L C S L A V L V P I V L F C E Q H V

62 TCGCGTTTCAGAGTGGCCAAGTTCTAGCTGCTCTTCCTAGAACCTCTAGGCAAGTTCAAG

19 F A F Q S G Q V L A A L P R T S R Q V Q

122 TTCTACAGAATCTTACTACAACATATGAGATTGTTCTCTGGCAGCCGGTAACAGCTGACC

39 V L Q N L T T T Y E I V L W Q P V T A D

182 TTATTGTGAAGAAAAAACAAGTCCATTTTTTTGTAAATGCATCTGATGTCGACAATGTGA

59 L I V K K K Q V H F F V N A S D V D N V

|  |  |
| --- | --- |
| 242 | AAGCCCATTTAAATGTGAGCGGAATTCCATGCAGTGTCTTGCTGGCAGACGTGGAAGATC |
| 79 | K A H L N V S G I P C S V L L A D V E D |
| 302 | TTATTCAACAGCAGATTTCCAACGACACAGTCAGCCCCCGAGCCTCCGCATCGTACTATG |
| 99 | L I Q Q Q I S N D T V S P R A S A S Y Y |
| 362 | AACAGTATCACTCACTAAATGAAATCTATTCTTGGATAGAATTTATAACTGAGAGGCATC |
| 119 | E Q Y H S L N E I Y S W I E F I T E R H |
| 422 | CTGATATGCTTACAAAAATCCACATTGGATCCTCATTTGAGAAGTACCCACTCTATGTTT |
| 139 | P D M L T K I H I G S S F E K Y P L Y V |
| 482 | *EcoRI*TAAAGGTTTCTGGAAAAGAACAAACAGCCAAAAATGCCATATGGATTGACTGTGGAATCC |
| 159 | L K V S G K E Q T A K N A I W I D C G I |
| 542 | ATGCCAGAGAATGGATCTCTCCTGCTTTCTGCTTGTGGTTCATAGGCCATATAACTCAAT |
| 179 | H A R E W I S P A F C L W F I G H I T Q |
| 602 | TCTATGGGATAATAGGGCAATATACCAATCTCCTGAGGCTTGTGGATTTCTATGTTATGC |
| 199 | F Y G I I G Q Y T N L L R L V D F Y V M |
| 662 | CGGTGGTTAATGTGGACGGTTATGACTACTCATGGAAAAAGAATCGAATGTGGAGAAAGA |
| 219 | P V V N V D G Y D Y S W K K N R M W R K |
| 722 | ACCGTTCTTTCTATGCGAACAATCATTGCATCGGAACAGACCTGAATAGGAACTTTGCTT |
| 239 | N R S F Y A N N H C I G T D L N R N F A |
| 782 | CCAAACACTGGTGTGAGGAAGGTGCATCCAGTTCCTCATGCTCGGAAACCTACTGTGGAC |
| 259 | S K H W C E E G A S S S S C S E T Y C G |
| 842 | TTTATCCTGAGTCAGAACCAGAAGTGAAGGCAGTGGCTAGTTTCTTGAGAAGAAATATCA |
| 279 | L Y P E S E P E V K A V A S F L R R N I |
| 902 | ACCAGATTAAAGCATACATCAGCATGCATTCATACTCCCAGCATATAGTGTTTCCATATT |
| 299 | N Q I K A Y I S M H S Y S Q H I V F P Y |
| 962 | CCTATACACGAAGTAAAAGCAAAGACCATGAGGAACTGTCTCTAGTAGCCAGTGAAGCAG |
| 319 | S Y T R S K S K D H E E L S L V A S E A |
| 1022 | *Spe*ITTCGTGCTATTGAGAAAACTAGTAAAAATACCAGGTATACACATGGCCATGGCTCAGAAA |
| 339 | V R A I E K T S K N T R Y T H G H G S E |
| 1082 | CCTTATACCTAGCTCCTGGAGGTGGGGACGATTGGATCTATGATTTGGGCATCAAATATT |
| 359 | T L Y L A P G G G D D W I Y D L G I K Y |
| 1142 | CGTTTACAATTGAACTTCGAGATACGGGCACATACGGATTCTTGCTGCCGGAGCGTTACA |
| 379 | S F T I E L R D T G T Y G F L L P E R Y |
| 1202 | TCAAACCCACCTGTAGAGAAGCTTTTGCCGCTGTCTCTAAAATAGCTTGGCATGTCATTA |
| 399 | I K P T C R E A F A A V S K I A W H V I |
| 1262 | *Age*I stopGGAATGTTACCGGTCATCATCACCATCACCATTGA |
| 419 | R N V T G H H H H H H |

The pcDNA4A plasmid vector encoding TAFI is 6387 bp, the TAFI DNA is 1275 bp and generates a protein 423 amino acids.

* To translate a DNA sequence into protein, you can use the Expasy Translation tool found at: <http://web.expasy.org/translate/>
* Paste the entire DNA sequence into the box and click on ‘translate sequence’
* The results generated will be 5’→3’ in each of the different possible reading frames (there are 3) and also 3’→5’
* Assess the sequences and look for open reading frames that begin with a start codon (MET) and terminates with the 6x-His (HHHHHH) followd by a stop codon. Depending on the length of the gene of interest, you may need multiple sequencing reactions to be able to determine sequence of the entire gene of interest (*i.e.* the 5’ end and the 3’ end)

When **designing primers for mutagenesis**, you need to keep in mind parameters including: melting temperature (°C), GC content (%), length (bp), 5’ flanking region, 3’ flanking region, what nucleotides will the primers terminate in, where the mutation is to be located, and if complementary primers or overlapping primers are to be designed, several factors need to be examined.

The *melting temperature* (T*m*) of primers is important because you need to keep in mind that there are two sets of primers that go into a PCR reaction and should be designed so that they have a similar T*m*. If they are mismatched then amplification may not work at all because the primer with the higher T*m* will mis-prime at lower temperatures and the primer with the lower T*m* may not work at higher temperatures. The *% GC content* is also important and should be in the range of 40 – 60 %. The GC pair is bound by three hydrogen bonds as opposed to AT pairs which only have two therefore, making GC pairs more stable. The primer sequence must also be chosen such that there are no PolyG or PolyC stretches that can promote non-specific annealing. In PCR reactions, the GC content is also used to predict the annealing temperature of the primers to the template DNA. Higher GC content indicates a higher T*m* and in turn higher annealing temperature. The DNA polymerase used in this lab (*Phusion* high fidelity) works at an optimal extension temperature of 72°C but also due to the higher salt concentrations in the buffer in which it is stored, makes it tend to work better at elevated denaturing and annealing temperatures. *Primer length* is

a critical factor for successful PCR reactions and works in conjunction with primer specificity and annealing temperatures. In general they should be between 20 – 40 bp in length making them extremely sequence specific and also giving rise to efficient annealing. If the primers are too short they could mis-match with the template DNA resulting in an incorrect mutation within the desired sequence. Primers that are too long may not properly anneal and with fewer templates primed, it can significantly decrease the amount of amplified product. When determining the *flanking regions*, the general rule of thumb is to have the mutation located in the center of your primers. The 3’ terminal position of the primers is critical to the control of mis-priming and it’s good to include a G or C residue at the 3’ end which essentially functions as a “clamp” due to the strong hydrogen bonds of the GC pair, ensuring correct binding at the 3’ end.

**Designing sequencing primers** on the other hand will be different as only one is needed as opposed to two. Sequencing primers must be able to anneal to the target DNA at a precise and unique location on a predictable strand of the newly created mutant DNA. A few things to keep in mind is that sequences are always written 5’ → 3’, DNA polymerase always extends from the 3’ end and the sequence which you will read will be *the same strand* as the *primer itself* (either sense or antisense). Sequencing primers should be between 18 – 30 nucleotides (nt), optimally 20 – 25 nt in length, have a GC content of 40-60 % and the Tm should be between 55 – 75 °C. If you are targeting the sequence to a specific region (ie. to identify a new mutation), position the primer so the desired sequence falls in the most accurate region. Sequence data is often most accurate around 80 - 150 nt away from the primer so don’t count on seeing good sequencing less than 50 nt away from the primer or more than 300 nt away.

### Procedure Overview:

*Day 1:* Site-directed mutagenesis (SDM) reaction, restriction digestion for plasmid mapping

*Day 2:* Transformation of SDM reaction, agarose gel electrophoresis of plasmid mapping digestions

*Day 3:* Minipreps of plasmid DNA from SDM colonies, determination of DNA concentration

*Day 4:* Restriction digestion and agarose gel electrophoresis of SDM minipreps

*2 Week (14 days) after Day 4* - Lab report due on Blackboard

### Procedures:

*Day 1*

### Important information:

You will be provided with an aliquot of 200 ng/µl ‘wild-type’ TAFI pcNDA.4A. This is your plasmid DNA for the restriction digest as well as template for your PCR-based site-directed mutagenesis. Pay attention to the concentration of plasmid DNA required for both of these independent experiments.

***Note:*** For part B, the site-directed mutagenesis, you will need template plasmid concentration at 10 ng*/*µl. You will need to do the calculation and dilution before setting up the reaction for Part B. If you don’t dilute your plasmid DNA, or dilute it incorrectly, your site-directed mutagenesis will not work.

Part A: Restriction digest of a TAFI expression plasmid

1. Assemble a series of restriction digests in 1.5 ml microcentrifuge tubes as follows:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *Component* | **Control** | **Reaction 1** | **Reaction 2** | **Reaction 3** |
| *Volume (μl)* |
| **1** | H2O | 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* |

\*\* Plasmid DNA is ‘wild-type’ TAFI pcNDA.4A. Total amount per reaction is 1 µg \*\*

1. Assemble each reaction on ice in the exact order listed.

Once all components have been added, mix each reaction thoroughly by flicking the tube. Collect the fluid in the bottom of the tube by brief (1 sec) centrifugation.

1. Incubate the reactions in a 37°C water bath for 1 hour. During the incubation proceed to start on part B
2. At the conclusion of the incubation, add 4 μl loading dye (6xLD) to each reaction and mix. The reactions will be stored at -20°C until the next lab session, where you will subject them to electrophoresis. Make sure you can identify your samples.

Part B: Site-directed mutagenesis

1. You will be provided with one 0.2-mL PCR microcentrifuge tube containing 1 μl of dNTP (10 mM each) mix. Assemble the remainder of the reaction in this tube as follows:

7.5 μl H2O

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

* the mutagenesis protocol uses 125 ng total of each oligonucleotide primer \*
1. Mix the reaction well by flicking the tube; collect the fluid in the bottom of the tube by brief (1 sec) centrifugation.
2. Place the reactions in the thermal cycler. It has been programmed as follows: Segment 1 – Initial denaturation

Segment 2- [Denaturation, annealing, elongation] repeat Segment 3 – Final elongation

|  |  |  |  |
| --- | --- | --- | --- |
| **Segment** | **Cycles** | **Temperature** | **Time** |
| 1 | 1 | 98°C | 30 sec |
| 2 | 25 | 98°C | 10 sec |
| 60°C | 30 sec |
| 72°C | 6 min |
| 3 | 1 | 72°C | 6 min |

1. The reactions, once completed, will be stored at -20°C until the next lab session, when you will complete the procedure.

# END OF LAB DAY 1

*Day 2*

Part C: *Dpn*I digestion and bacterial transformation of site-directed mutagenesis reaction

### Start right away at the beginning of the lab so that you don’t run out of time

\*\*NOTE: The *E. coli* bacteria used are a Biosafety Level 1 hazard. Be sure to wear gloves when handling bacteria or bacterial media and dispose of solid waste in the biohazard waste containers\*\*

1. Add 1 μl of *Dpn*I restriction enzyme (to digest the template DNA present after performing SDM PCR) and gently mix. Collect the fluid in the bottom of the tube by brief (1 sec) centrifugation. Incubate in the 37°C incubator for 1 hour.
2. You will be supplied with a 50-μl aliquot of thawed XL-1 Blue super-competent *E. coli* in a pre-chilled 1.5-ml microcentrifuge tube.
3. Transfer 10 μl of the *Dpn*I-treated mutagenesis reaction into the bacterial cells.
4. Mix very gently and incubate on ice for 30 minutes.
5. Heat-pulse the mixture for exactly 45 seconds in a 42°C heat block and then place the reactions on ice for 2 minutes.
6. Add 0.5 ml of LB broth (pre-heated to 37°C) and incubate the transformation reactions at 37°C for 30 minutes.
7. Centrifuge your tubes at 6,000 rpm for 1 minute room temperature
8. Remove all but 100 µl of the liquid and dispose of in the proper label container in the fume hood.
9. Resuspend the bacterial pellet and plate the entire volume ~ 100 μL of the mixture onto a pre-warmed LB-agar plate containing 100 μg/mL ampicillin. Using a sterile L-shaped spreader, spread the mixture on the plate until the fluid has almost disappeared. Place the plates upside-down in a 37°C incubator overnight. \*\*The coordinator will remove them the following day, parafilm the plates and store inverted at 4˚C \*\*

Part D: Agarose gel electrophoresis of plasmid restriction digests.

\*\* NOTE: Due to the presence of Ethidium Bromide in the gel and gel buffer, you MUST WEAR GLOVES!!!! Ethidium bromide is a carcinogen!! \*\*

1. Retrieve your four reactions as well as an aliquot of 1 kb ladder (Frogga Bio), which will serve as a marker on the gel.
2. Acquire one pre-made 1% agarose gel and carefully transfer it into the tray of your agarose gel apparatus. NOTE: the wells should be near the black end so that your samples will migrate towards the red leads (remember DNA is negatively charged and will therefore migrate towards the anode - red). Gently pour 1×TAE running buffer into the gel apparatus until your agarose gel is submerged approximately 3-6 mm under the buffer.
3. Using your P20 pipet, load 10 µl (entire aliquot) of the 1 kb DNA ladder (Frogga Bio) into lane 1 of your gel, then using P200 load 24 μl of the four digests into four consecutive separate lanes, as shown below:

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

1. Close the lid of the gel tank and attach the electrical leads. Before you turn the power supply on, ask GA/TA to check it. Turn on the power supply and apply a voltage of 80V. Run your gel for approximately 45 minutes. Check the migration of your samples by visualizing the migration of the purple dye front. Once the purple dye front has migrated to within 3 cm of the bottom of the gel, turn off the power supply and remove the electrical leads. Inform your GAs that you are prepared to image your gel.
2. Examine and acquire an image of your gel by UV excitation using the Syngene imaging system. Have GA/TA save your image for later upload in the results folder on Blackboard.
3. Discard your gel into labelled container in the fume hood, wash your gel running apparatus and clean up your work station.

# END OF LAB DAY 2 – END OF WEEK 1

*Day 3*

Part E: Miniprep of plasmid DNA

\*\*NOTE: The *E. coli* bacteria used are a Biosafety Level 1 hazard. Be sure to wear gloves when handling bacteria or bacterial media and dispose of solid waste in the biohazard waste containers\*\*

1. The lab coordinator will have prepared two – 3 mL overnight cultures in LB (containing 100 μg/ml ampicillin) from two colonies that grew on your LB-agar plates following transformation of your mutagenesis reaction on lab day 2.
2. Transfer approximately 1 ml of the overnight culture into a 1.5-ml microcentrifuge tube.
3. Centrifuge at 6,000 rpm for 1 minute and remove as much of the supernatant as possible with a P1000 pipet. Be sure not to disturb the bacterial pellet.
4. Repeat steps 2-3 two more times (discarding the supernatant after each spin) to have a total bacterial pellet yield from 3 ml of your overnight cultures.
5. Resuspend the bacterial pellet in 200 μl of Resuspension Solution (S1) by pipetting up and down. Be sure not to draw any fluid into the barrel of the P200.
6. Add 200 μl of Lysis Solution (S2). Invert gently to mix. Incubate for 5 minutes at room temperature; you should see the fluid clarify.
7. Add 350 μl of Neutralization Solution (S3). Shake vigorously to mix; an extensive white precipitate should appear that contains the bacterial genomic DNA, while the plasmid DNA remains soluble.
8. Centrifuge at maximum speed for 10 minutes to pellet the debris. Transfer the supernatant containing the plasmid DNA into a fresh tube; repeat centrifugation if necessary to remove any remaining precipitate.
9. For each preparation, place a binding column into a collection tube. Add 500 μl Column Preparation Solution to binding column, then centrifuge at maximum speed for 1 minute. Discard flow-through.
10. Transfer cleared lysate into binding column, then centrifuge 1 minute. *The DNA remains bound to the column*. Discard flow-through.
11. Add 750 μl Wash Solution to the column, then centrifuge for 1 minute. *The DNA remains bound to the column*. Discard flow-through.
12. Centrifuge the column once again for 1 minute to dry the column.
13. Transfer column to a new collection tube. Add 50 μl nuclease free H2O, then centrifuge at maximum speed for 1 minute. The plasmid DNA elutes from the column and is located in the fluid in the collection tube. Discard the column and save the plasmid DNA for subsequent analysis.

Part F: Determination of plasmid DNA concentration

1. Go to the Nanodrop station along with your GAs. Open the Nanodrop software and select the nucleic acid module.
2. Carefully add 2 µl of nuclease free water to the center of the pedestal and carefully close the sampling arm. Click on 'ok' to initialize the spectrometer. A message "Initializing Spectrometer - please wait" will appear. Once this message on the screen disappears, the unit is now ready for use.
3. Gently wipe the pedestal with a kimwipe and add 2 µl of nuclease free H2O to the center of the pedestal. Close the arm and click on "BLANK". A measurement will be taken and a straight line will appear on the graph. Wipe the pedestal and arm with a kimwipe.
4. To the center of the pedestal, carefully add 2 µl of your DNA sample. Analyze the concentration of your DNA by clicking on the "measure" button. Record the concentration of your sample (ng/µl) and purity (260/280) and include all relevant information in your lab report.

\*Conc of sample A = 196.5 ng/µl Purity of sample A = 1.86

 Conc of sample B = 114.3 ng/µl Purity of sample B = 1.88

1. Calculate the volume of your mini-prepped plasmid DNA you would need to use to prepare 10μl of 200 ng/μl final concentration. If you are not sure how to do this calculation, ask your GA/TA (HINT: C1V1 = C2V2). Prepare a sample of your DNA at 200 ng/ul which will be used on day 4 for restriction digest. Store both of your plasmid DNAs (concentrated and diluted) at -20˚C.

\* The DNA concentration was too low, so it did not dilute. Therefore, restriction analyse was reduced to get 200ng/μl on final concentration.

# END OF LAB DAY 3

*Day 4*

Part G: Restriction analysis of clones from site-directed mutagenesis

1. Retrieve your two minipreps that you prepared yesterday. Assemble AvrII/SpeI double digests for each clone as you did in Part A *(i.e.* one digestion reaction per clone). In addition, assemble one double digest (same as reaction 3 in the chart on page 3) with the “wild-type” TAFI-pcDNA4A (200 ng/µl) plasmid as a positive control. \*\* NOTE: you should have 1 µg total DNA for each of your reactions. If your clone DNA concentrations are slightly under 200 ng/µl you can adjust the volume of H2O to account for this, *i.e.* if you need 7 µl of your DNA you would then use only 10 µl of H2O \*\* Have your GAs check your calculation before proceeding.

|  |  |  |  |
| --- | --- | --- | --- |
| *Component* | **Control** | **Clone #1** | **Clone #2** |
| *Volume (μl)* |
| **1** | H2O | 12 | 12  | 12 (3.25) |
| **2** | 10× CutSmart buffer | 2 | 2 | 2 |
| **3** | Plasmid DNA | 5 | 5 | 5 (8.7) |
| **4** | AvrII | 0.5 | 0.5 | 0.5 |
| **5** | SpeI | 0.5 | 0.5 | 0.5 |
|  | *Total volume* | *20* | *20* | *20* |

\*The values in red were used for Clone #2 instead of the ones in black because the DNA concentration was too low, so it did not dilute. Therefore, restriction analyse was reduced to get 200ng/μl on final concentration.

1. Incubate for 1 hour at 37°C in the incubator. At the conclusion of the incubation, add 4 μl loading dye to each sample and mix. Collect your samples at the bottom of your tube by brief centrifugation (1 sec).
2. Retrieve a pre-made 1% agarose gel from your GAs, a positive clone control digest and wild type digest aliquots that already have loading dye added to them (this is a positive mutagenesis sample and wild type DNA that have both been double digested with AvrII/SpeI for you by the lab coordinator). Gently pour 1× TAE running buffer into the gel apparatus until your agarose gel is submerged approximately 3-6 mm under the buffer.
3. Load your samples in the following order:
	1. 1 kb ladder (10 μl)
	2. Positive wild-type control digest (load entire sample) \*given to you\*
	3. Positive clone control digest (load entire sample) \* given to you \* - (has mutation)
	4. Your experimental control digest (24 µl)
	5. Digest of clone 1 (24 μl)
	6. Digest of clone 2 (24 μl)
	7. 6-8. Empty
4. Set-up the gel as previously, ask your GA/TA to check and turn the power-supply on. While your gel is running you can work on data analysis from previous days – if you have any questions or need some help, this is a good time to ask you GA and/or TA.
5. Inform your GAs that you are prepared to image your gel.
6. Examine and acquire an image of your gel by UV excitation using the Syngene imaging system. Have GA/TA save your image for later upload in the results folder on Blackboard.
7. Discard your gel into labelled container in the fume hood, wash your gel running apparatus and clean up your workstation.

# END OF LAB DAY 4 – END OF WEEK 2

## References and Further Reading:

1. Watson, Baker, Bell, Gann, Levine, Losick. Molecular Biology of the Gene 6th edition. Pearson, 2008. Part 5 (Chapters 21, 22).
2. Voet, Voet & Pratt. Fundamentals of Biochemistry 4th edition. Wiley, 2013. Chapter 3.
3. Brown. Biotechnology, A Laboratory Skills Course 1st edition. Bio-Rad, 2011.
4. Boffa MB, Wang W, Bajzar L, Nesheim ME. Plasma and recombinant thrombin-activable fibrinolysis inhibitor (TAFI) and activated TAFI compared with respect to glycosylation, thrombin/thrombomodulin-dependent activation, thermal stability, and enzymatic properties. *J Biol Chem.* 1998; 273:2127-35.
5. Schneider M, Boffa M, Stewart R, Rahman M, Koschinsky M, Nesheim M. Two naturally occurring variants of TAFI (Thr-325 and Ile-325) differ substantially with respect to thermal stability and antifibrinolytic activity of the enzyme. *J Biol Chem.* 2002; 277:1021-30.
6. Boffa MB, Reid TS, Joo E, Nesheim ME, Koschinsky ML. Characterization of the gene encoding human TAFI (thrombinactivable fibrinolysis inhibitor; plasma procarboxypeptidase B). Biochemistry 1999; 38:6547-58.