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A report submitted

upon completion of summer project

on

Due Ferric Single chain (DFSc) gene cloning

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CERTIFICATE OF COMPLETION

This is to certify that **Ms. M. Adishree** of NISER, has successfully completed the summer project for the academic year of 2016 in the institution **NISER**, Jatni, Khordha.

Chairperson

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ACKNOWLEDGEMENT

I wish to express my sincere gratitude to Dr. Rudresh Acharya for providing me an opportunity to work under him.

I sincerely thank Mr. Ramakrushna Haibru for his guidance and encouragement in carrying out this project. I admire him for his earnest efforts to make this project a success.

I also wish to express my gratitude to Ms. Barsa Tripathy for her support.

I would like to thank my friends and family for their endless love and support in the long run of my life.

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INTRODUCTION

- Initial design of diiron peptides evolved from a retro-structural analysis of the six different carboxylate-bridged diiron proteins.
- The first two subsets of the DueFerri family (DF), DF1 and DF2, contain two noncovalently associated helix–loop– helix motifs, which bind the dimetal cofactor near the center of the structure.
- Our design evolved with the construction of heterotetramers, DFtet, consisting of four disconnected helices. These helices can be combinatorially assembled to create a large number of helical bundles.
- Finally, using a computational approach, the feasibility of constructing a monomeric four-helix bundle was explored, encompassing the dimetal cofactor, DFsc.

DFsc: MDELRELLKA EQQGIKILKE VLKKAKEGDE QELARLNQEI VKAEKQGVKV YKEAAEKARN PEKRQVIDKI LEDEEKHIEW HKAASKQGNA EQFASLVQQH LQDEQRHVEE IEKKN

GENE DESIGNING

DNA insert was designed with our desired protein.

Gene

5’-Random- BamHI-NcoI-FactorXa-DFsc-Stop-EcoRI- HindIII-Random-3’

Sequence

01AAAAAAGGATCCCCATGGATTGAAGGCCGCATGGATGAACTGCGTGAGCTGCTGAAGGCG
61 GAACAGCAGGGAATTAAGATTCTGAAAGAAGTACTGAAAAAAGCGAAGGAAGGCGACGAA
121 CAGGAACTGGCGCGTCTGAATCAGGAAATTGTGAAAGCGGAAAAGCAGGGCGTGAAAGTG 181
TATAAAGAAGCGGCAGAGAAGGCGCGCAATCCGGAAAAACGTCAGGTGATTGATAAAATC
241 CTGGAAGATGAAGAAAAACATATTGAATGGCATAAAGCGGCGAGCAAACAGGGCAATGCA
301 GAACAGTTTGCGTCACTGGTGCAGCAGCATCTGCAGGATGAGCAGCGTCATGTGGAAGAA
361 ATTGAGAAAAAGAACTAAGAATTCAAGCTTAAAAAA

- **Reverse translation** is inferring DNA sequence from the amino acid sequence of a protein.
- Reverse translation is often employed to make a hybridization probe or a PCR primer used to clone the gene encoding the protein of interest.
- Determining the DNA or mRNA sequence coding for a specific amino acid, is more complex because the **genetic code is "degenerate“** . Reverse translation of a protein does not produce a single nucleotide sequence. Instead, it results in a population of different sequences that, if translated, would all code for the same amino acid sequence.
- To identify the actual genomic sequence that codes for the protein in vivo, it is necessary to clone and sequence the gene for the protein.
- The first step in cloning is to synthesize a mixture of oligonucleotides (oligos) that corresponds to all of the potential coding sequences determined by reverse translation. This pool of oligos is used as a "degenerate" (mixed) hybridization probe to isolate the corresponding DNA or cDNA clone from a library.
- Different organisms preferentially use particular codons to specify amino acids, and this **codon usage bias** should also be taken into account when designing oligos by reverse translation.

VECTOR MAPS:

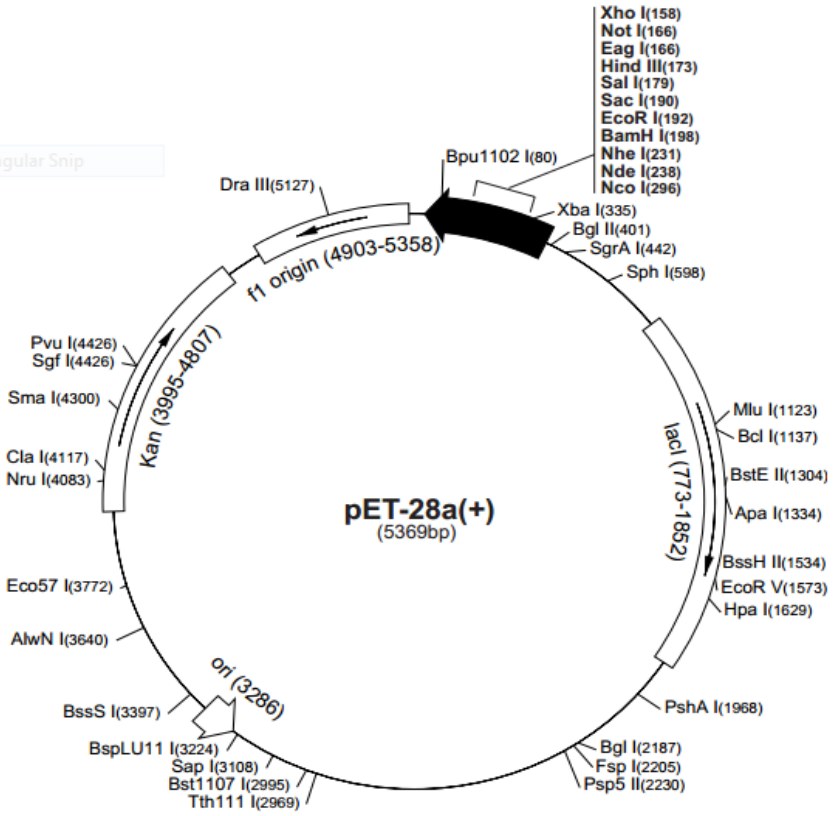


Figure 1: pET-28A(+) vector map

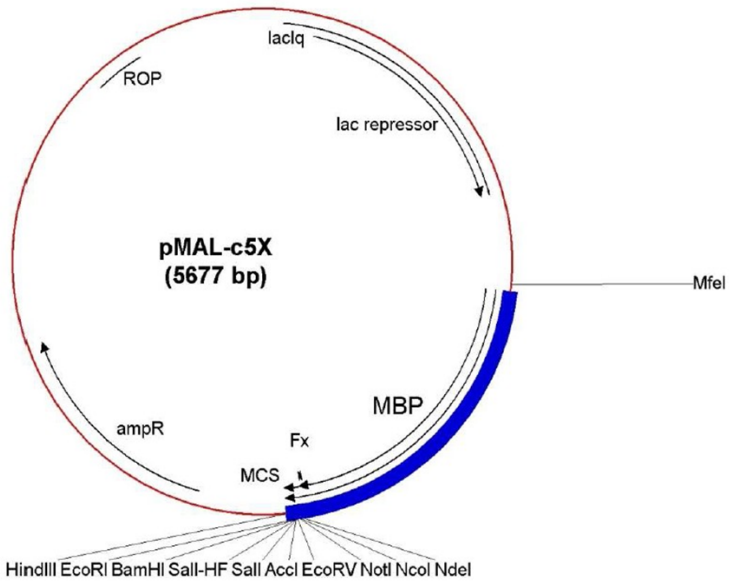


Figure 2: pMAL-c5X vector map

DNAWorks (https://hpcwebapps.cit.nih.gov/dnaworks/)

automates the design of oligonucleotides for gene synthesis by PCR-based methods. It only requires simple input information, i.e. amino acid sequence of the target protein and melting temperature (needed for the gene assembly) of synthetic oligonucleotides. The program outputs a series of oligonucleotide sequences with codons optimized for expression in an organism of choice.

Oligos

```
01  AAAAAAGGATCCCCATGGATTGAAGGCCGCATGGATGAACTGCGTGAGCTGC                52
02  GTACTTCTTTCAGAATCTTAATTCCCTGCTGTTCCGCCTTCAGCAGCTCACGCAGTTCAT        60
03  GGGAATTAAGATTCTGAAAGAAGTACTGAAAAAAGCGAAGGAAGGCGACGAACAGGAACT 60
04  CCCTGCTTTTCCGCTTTCACAATTTCCTGATTCAGACGCGCCAGTTCCTGTTCGTCGCCT        60
05  TGAAAGCGGAAAAGCAGGGCGTGAAAGTGTATAAAGAAGCGGCAGAGAAGGCGCGCAATC 60
06  TCTTCATCTTCCAGGATTTTATCAATCACCTGACGTTTTTCCGGATTGCGCGCCTTCTCT        60
07  TTGATAAAATCCTGGAAGATGAAGAAAAACATATTGAATGGCATAAAGCGGCGAGCAAAC        60
08  ATGCTGCTGCACCAGTGACGCAAACTGTTCTGCATTGCCCTGTTTGCTCGCCGCTTTATG        60
09  CACTGGTGCAGCAGCATCTGCAGGATGAGCAGCGTCATGTGGAAGAAATTGAGAAAAAGA 60
10  TTTTTTAAGCTTGAATTCTTAGTTCTTTTTCTCAATTTCTTCCACATG                    48
```

	position (pos)	**Length (len)**	**melting temperature (Tm)**	**Amp.**
Forward primer ATGGATGAACTGCGTGAGCT	30	20	62.40	
AAAAAGGATCCCCATGGA		18	56.12	
TCAAGAAAAAGAGTTAAAGAAGGTGTAC		28	57.83	
Reverse primer TTCTCAATTTCTTCCACATGACGC	367	24	62.39	337

DNA AMPLIFICATION

Designed insert was amplified by Polymerisation Chain Reaction (PCR).

Principle:

- PCR amplifies a specific region of a DNA strand (the DNA target).
- The method relies on **thermal cycling**, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA.
- **Primers** (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase, which the method is named after, are key components to enable selective and repeated amplification.
- As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses.
- Almost all PCR applications employ a **heat-stable DNA polymerase, such as Taq polymerase** (an enzyme originally isolated from the bacterium *Thermus aquaticus*).

Components and reagents required for PCR:

1. **DNA template** that contains the DNA region to amplify.
2. **Two primers** that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target.
3. **Taq polymerase**
4. **Deoxynucleoside triphosphates** (dNTPs; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand.
5. **Buffer solution**, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase
6. **Bivalent cations**, magnesium or manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be used for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis.
7. **Monovalent cation**- potassium ions

8. The PCR is commonly carried out in a reaction volume of 10–200 µl in small **reaction tubes** (0.2–0.5 ml volumes) in a thermal cycler.
    - ➔ Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration.
    - ➔ Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube.

No.	Ingredient	Volume (in µl)
1.	2 x mix	12.5µl
2.	10x buffer	2.5µl
3.	Taq polymerase	1µl
4.	Template	5µl
5.	Primer	2µl
6.	AMQ	2µl
		Total = 25 µL

Procedure:

PCR consists of a series of 20–40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2–3 discrete temperature steps, usually three:-

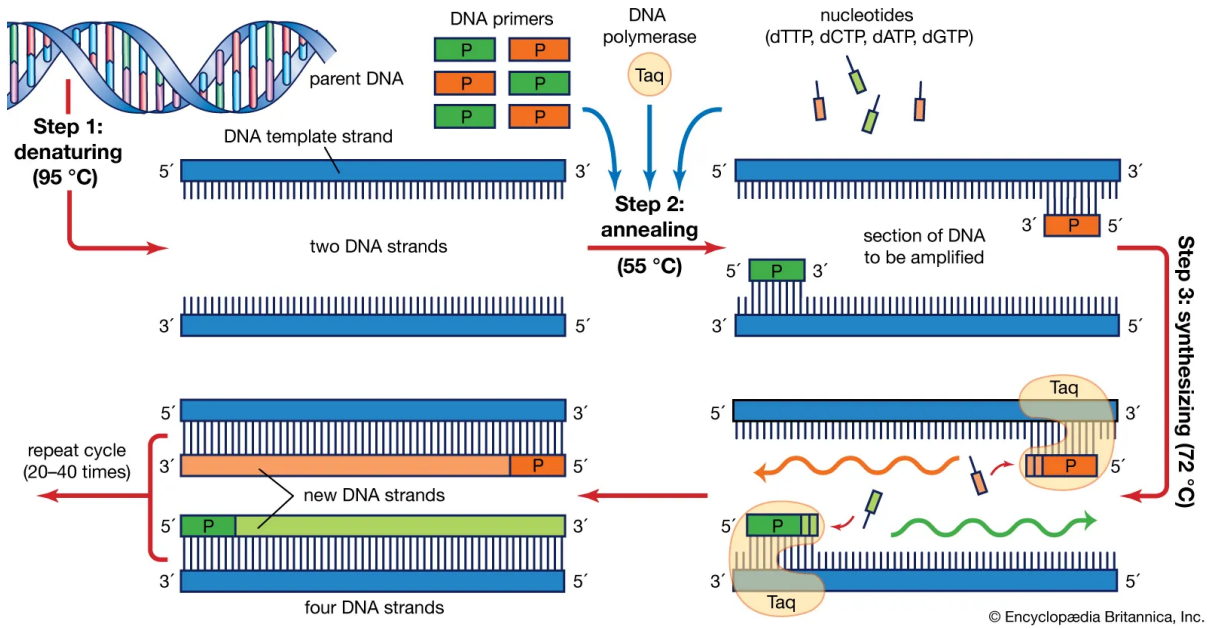
Step 1: Initialization (heat activation by hot-start PCR):
it consists of heating the reaction to a temperature of 98 °C, held for 30 seconds.

Step 2: Denaturation

This step is the first regular cycling event and consists of heating the reaction to 98 °C for 10 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Step 3: Annealing

The reaction temperature is lowered to 62°C for 15 seconds allowing annealing of the primers to the single-stranded DNA template. The temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hy hybridization to be specific.



Step 4: Extension/elongation

The temperature at this step depends on the DNA polymerase used; for Taq polymerase a temperature of 72 °C is used. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand.

Step 5: Final elongation

This single step is performed at a temperature of 72°C for 5 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Step 6: Final hold

This step at 4°C for an indefinite time is employed for short-term storage of the reaction.

GEL ELECTROPHORESIS

A gel run was done to elute the amplified DNA.

Principle:

- Gel electrophoresis uses a gel as an anticonvective medium and/or sieving medium during electrophoresis, the movement of a charged particle in an electrical field.
- Gels suppress the thermal convection caused by application of the electric field, and can also act as a sieving medium, retarding the passage of molecules.
- Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through a matrix of agarose or other substances.
- Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving.
- Proteins are separated by charge in agarose because the pores of the gel are too large to sieve proteins.

Procedure:

1. 50ml buffer (1x TAE) was taken. (some extra ml of buffer was taken to even up the amount of buffer lost in heating the solution)
2. 1% agarose powder was added.
3. The mixture was heated up till it's a clear solution.
4. 3µL Ethidium bromide (EtBr) was added to the solution. EtBr acts as a staining reagent.
5. The solution was immediately poured into gel caster before it solidifies in the container itself.
6. waited till the wells are visible.
7. The amplified DNA samples, vectors PMALc5x & PET28a and the ladder were loaded in the wells and the apparatus was kept undisturbed for 15-20 minutes. (When kept at high voltage of 111V)
8. Coloured bands were seen. The gel was then observed under Gel Docx.

ELUTION

The vectors and insert were extracted from the gel.

After DNA samples are run on an agarose gel, extraction involves four basic steps:

I. identifying the fragments of interest,
II. isolating the corresponding bands,
III. isolating the DNA from those bands, and
IV. removing the accompanying salts and stain.

- To begin, UV light is shone on the gel in order to illuminate all the ethidium bromide-stained DNA.
- Care must be taken to avoid exposing the DNA to mutagenic radiation for longer than absolutely necessary.
- The desired band is identified and physically removed with a scalpel. The removed slice of gel should contain the desired DNA inside.

Protocol:

1. Gel slice weighed in a colorless tube.
2. (Volume of gel x3) QG buffer added.
3. Incubated at 50°C for 10 min (or until the gel slice has completely dissolved).
4. Added 1 gel volume of isopropanol to the sample and mix.
5. Sample transferred to spin column and centrifuged for 1min.
6. Flow-through discarded and dry spin done.
7. Spin column placed into a clean 1.5 ml microcentrifuge tube**.**
8. Microcentrifuge tubes and EB tube kept in heat block at 55℃ for 5min.
9. Added 50 µl of Buffer EB to the center of the membrane and centrifuged the column for 1min at maximum speed. (Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the membrane, let the column stand for 1 min, and then centrifuge for 1 min.)

DNA QUANTIFICATION

Concentration and purity of DNA was measured using nanodrop for further calculations.

- Quantitation of nucleic acids is commonly performed to determine the average concentrations of DNA or RNA present in a mixture, as well as their purity.
- Reactions that use nucleic acids often require particular amounts and purity for optimum performance.
- **Spectrophotometric analysis** is based on the principles that nucleic acids absorb ultraviolet light in a specific pattern. In the case of DNA and RNA, a sample that is exposed to ultraviolet light at a wavelength of 260 nanometres (nm) will absorb that ultraviolet light.
- Light in the ultraviolet (UV) and visible (vis) range of the electromagnetic spectrum shows an energy that is equivalent to about 150 to 400 kJ/mol.
- Light with the appropriate energy is used to promote electrons from the ground state to an excited state.
- The absorption of energy from the incident light as a function of its wavelength is measured in absorption spectroscopy.
- Nucleic acids absorb ultraviolet (UV) light due to the heterocyclic rings of the nucleotides; the sugar-phosphate backbone does not contribute to absorption.
- The wavelength of maximum absorption for both DNA and RNA is 260nm (**λmax = 260nm**) with a characteristic value for each base.

Base	pH	λmax nm
Adenine	1	262.5
	7	260.5
	12	269
Cytosine	1	276
	7	267
	14	282
Guanine	1	276
	7	276
	11	274
Thymine	4	264
	7	264
	12	291

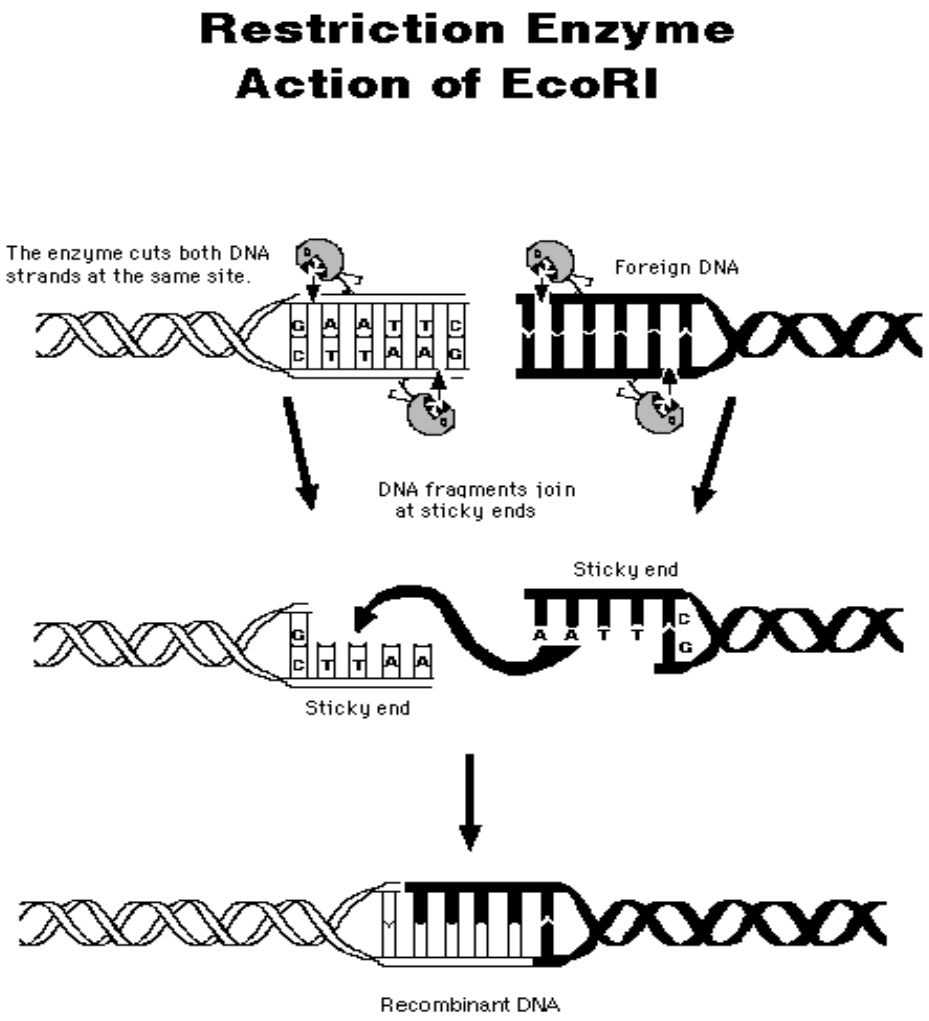
RESTRICTION DIGESTION

The vectors PMALc5x & PET28a were cut by EcoRI and NcoRI enzymes.

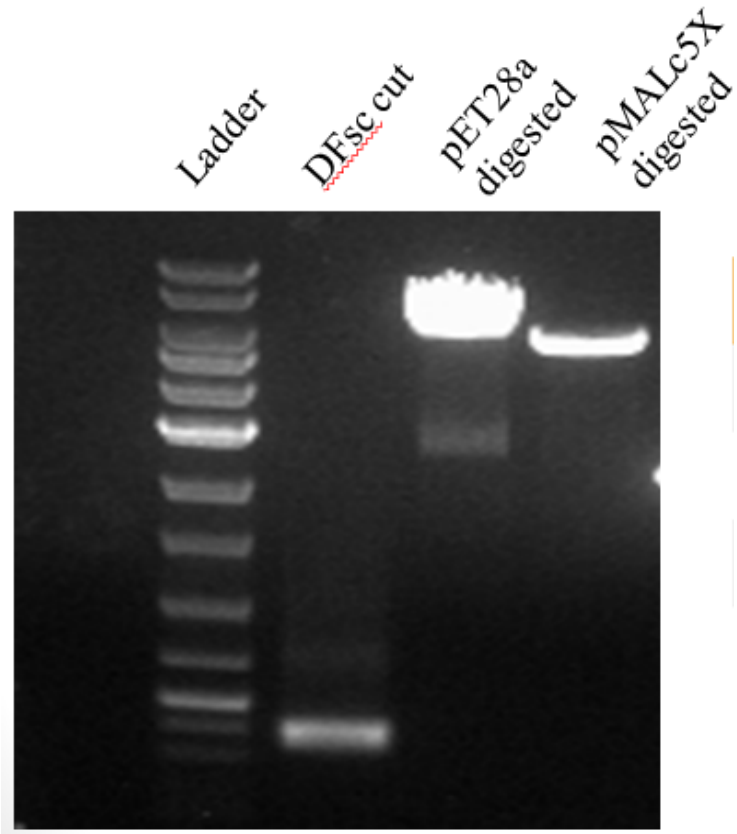
A gel run was done to check the cut vector(s). If found uncut, the process is repeated.

EcoRI	*Escherichia coli* RY13	5' GAATTC 3' CTTAAG	5' ---G AATTC--- 3' 3' ---CTTAA G--- 5

NcoI	*Nocardia corallina*	5' CCATGG 3' GGTACC	5' ---C CATGG--- 3' 3' ---GGTAC C--- 5'



S.No.		DFSc	
		PET28a(µl)	PMAL(µl)
1.	Vector	6	3
2.	Insert	1	1
3.	2 x Ligase	10	10
4.	Ligase	1	1
5.	AMQ	2	5
	Total	20µl	20µl



	Concentration(ng/µl)	purity
DFsc	25.9	2.2
PMAL	32.4	2
PET28a	16.2	2.4

LIGATION

Cut vector and insert were ligated following the ligation protocol.

Components and reagents required for ligation:

- Cut vector DNA
- Insert (volume of vector and insert as calculated by ligation calculator from the measured concentration)
- Ligase buffer
- Ligase
- Milli-Q water

Protocol:

1. All the requisite components and reagents were added up and mixed thoroughly.
2. centrifuged briefly and incubated at room temperature for 5 minutes.
3. chilled on ice and then stored at -20° C.

15 minutes ligation reaction done.

S.No.		DFSc		ABC	
		PET28a(µl)	PMAL(µl)	PET28a(µl)	PMAL(µl)
1.	Vector	6	3	7	3
2.	Insert	1	1	2	2
3.	2 x Ligase	10	10	10	10
4.	Ligase	1	1	1	1
5.	AMQ	2	5	0	4
	Total	20µl	20µl	20µl	20µl

TRANSFORMATION

Transformation is the genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material (exogenous DNA i.e. our insert here) from its surroundings through the cell membrane.

Components and reagents needed:

- Antibiotics
- **E-coli competent cells**
- Lb broth

Note: PMALc5x is resistant to Ampicillin and PET28a is resistant to Kanamycin.

Protocol:

1. Ampicillin and Kanamycin added to PMALc5x and PET28a respectively.
2. Vectors then added to E-coli competent cells in separate tubes.
3. Tubes stored in ice block for 30 minutes.
4. Then 2 minutes heat shock in water bath at 37° C
5. Again kept in ice for 2 minutes.
6. 950µL lb broth added to each tube.
7. Kept in incubator for 40 minutes (37° C, 220rpm)
8. As 1 generation of E-coli cells take 20 minutes to grow, hence 40 minutes for 2 such generations.
9. 2 min. short spin at 4000rpm, so that the cells are pelleted down.
10. 800µl broth taken out from each tube and cells were resuspended.

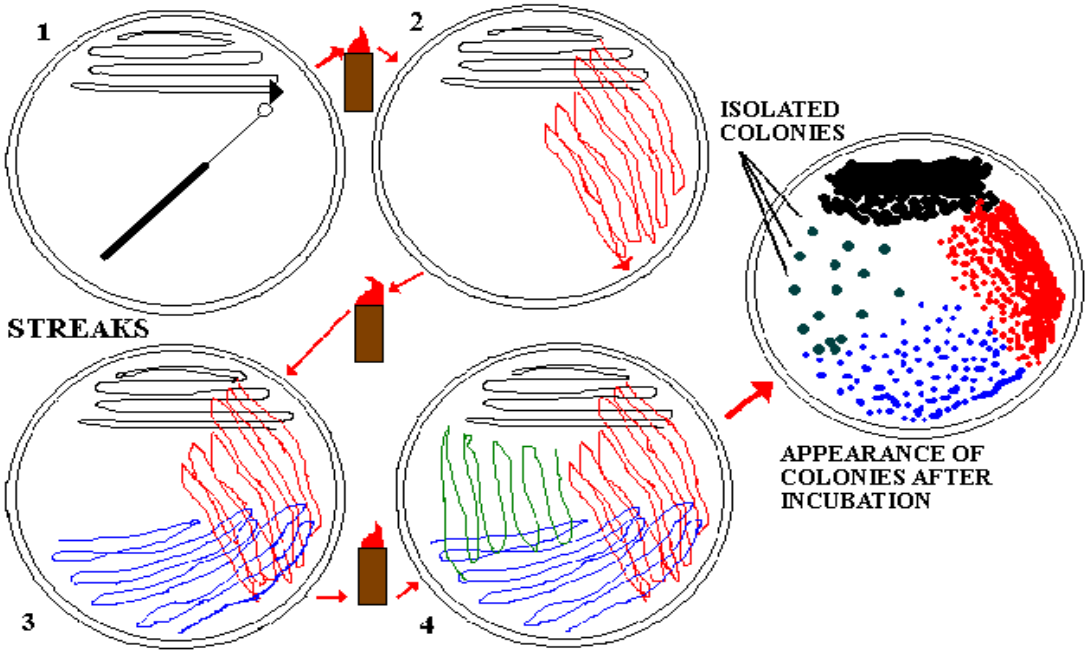
PLATING AND STREAKING

- Plasmids can carry one or more **antibiotic resistance genes**, which confer resistance to a specific antibiotic to the bacteria carrying them.
- The presence of an antibiotic resistance gene on a plasmid allows researchers to easily isolate bacteria containing that plasmid from bacteria that do not contain it by artificial selection (i.e. growing the bacteria in the presence of the antibiotic).
- **Luria broth (LB)** is a nutrient-rich media commonly used to culture bacteria in the lab.
- The addition of agar to LB results in the formation of a gel that bacteria can grow on, as they are unable to digest the agar but can gather nutrition from the LB within.
- The addition of an antibiotic to this gel allows for the selection of only those bacteria with the specific antibiotic resistance usually conferred by a plasmid carrying the antibiotic resistance gene.

The following protocol is for making LB agar plates for the purpose of bacterial selection:

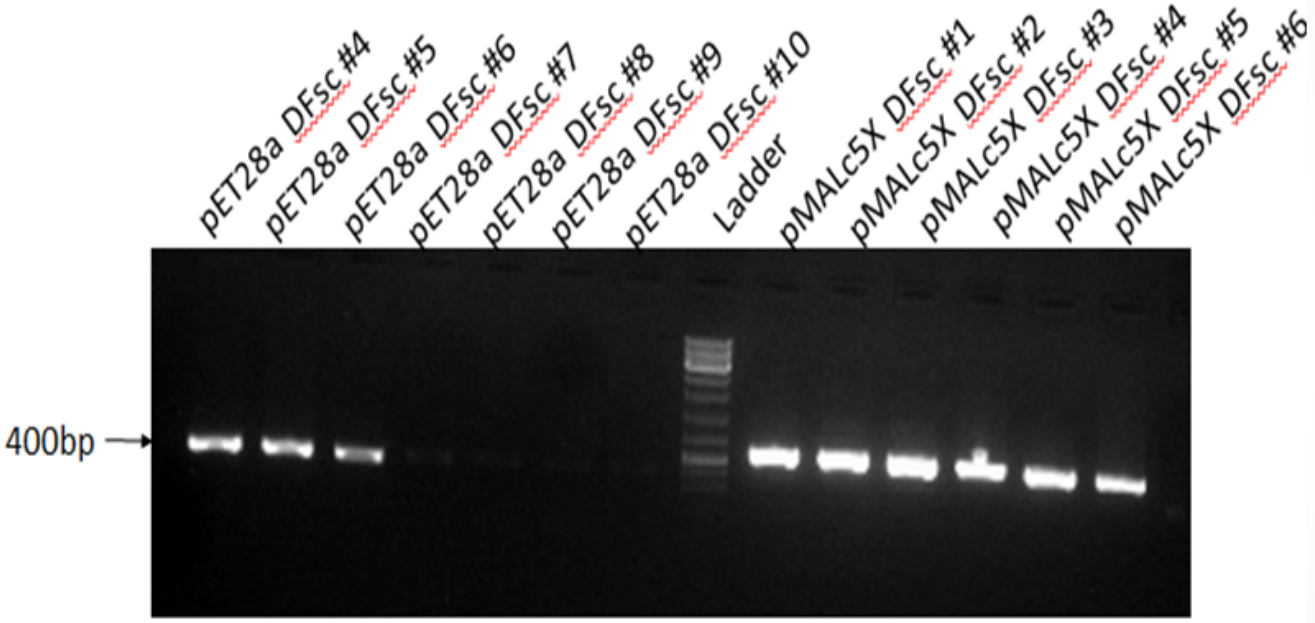
1. Required amount of LB agar powder weighed out into an Erlenmeyer flask.
2. Water added to the flask and stirred well.
3. Covered the top of the flask with aluminium foil and labelled with autoclave tape.
4. Autoclaved on the liquid setting for 15 minutes.
5. Allowed the agar solution to cool to 55°C by placing in oven, after removing the solution from the autoclave.
6. Added appropriate amount of desired antibiotic to the solution (100µg/ml ampicillin and 50µg/ml kanamycin) and swirled to mix.
7. Poured the agar into the petri dish slowly (inside hood).
8. Covered the plates with lids and allowed to cool for 30-60 minutes (until solidified).
9. Labelled the bottom of plates with antibiotic and date, sealed with parafilm and stored at 4°C.

- **Streaking** is a technique used to isolate a pure strain from a single species of microorganism, often bacteria. S
- amples can then be taken from the resulting colonies and a microbiological culture can be grown on a new plate so that the organism can be identified, studied, or tested.
- It is done by diluting a comparatively large concentration of bacteria to a smaller concentration.
- The decrease of bacteria should show that colonies are sufficiently spread apart to effect the separation of the different types of microbes.



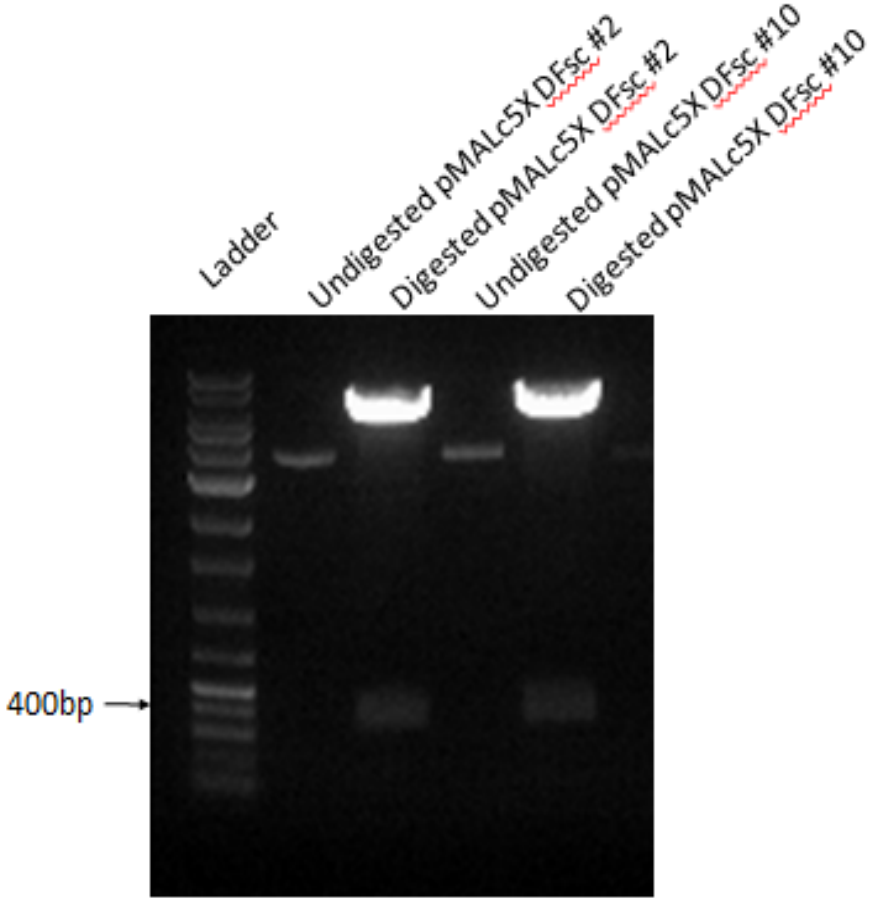
PRIMARY CULTURE

- Primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate (i.e., reach confluence).
- At this stage, the cells have to be subcultured (i.e., passaged) by transferring them to a new vessel with fresh growth medium to provide more room for continued growth.



Colony PCR

		D2	*D6*
1.	Template	20µl	20µl
2.	NcoI	3µl	3µl
3.	EcoRI	2.5µl	2.5µl
4.	10x cutsmart	2.5µl	2.5µl
5.	AMQ	2µl	2µl
	Total	30µl	30µl



PLASMID ISOLATION

Plasmid was isolated from the E-coli cells to check if they have the insert with our desired protein.

Protocol:

1. 1-5ml bacterial overnight culture pelleted by centrifugation at >8000rpm for 3min. at room temperature.
2. Pelleted bacterial cells resuspended in 250µl Buffer P1& transferred to a microcentrifuge tube.
3. 250µl Buffer P2 added & mixed thoroughly.
4. 350µl Buffer N3 added and mixed immediately.
5. centrifuged for 10 min. at 13,00rpm in a table top centrifuge.
6. Obtained supernatant transferred to separate spin columns and then centrifuged for 60s and discarded the flow-through.
7. Spin columns washed by adding 500µl Buffer PB and then centrifuged for 60s and discarded the flow-through.
8. Spin columns again washed by adding 700µl Buffer PE and then centrifuged for 60s and discarded the flow-through.
9. 1 min. dry spin done to remove residual wash buffer.
10. Spin columns placed in clean 1.5ml microcentrifuge tube. To elute DNA, 50 µl Buffer EB added to the center of each of the spin columns, let it stand for 1min, and centrifuged for 1 min.

Gel run was done to check for our insert.

PMAL	Concentration(ng/µl)	purity
D2	363	1.9
D6	340	1.9