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Preparing Plasmid Constructs to Investigate the Characteristics of Thiol Reductase and Flavin Reductase With Regard to Solubilizing Insoluble Proteinase Inhibitor 2 in Bacterial Protein Overexpression Systems

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Some proteins produced in bacterial expression systems can be insoluble and aggregated making them difficult to study. One possible solution to solubilizing such insoluble proteins is to create a fusion protein containing the protein of interest coupled with a thioreductase protein. (Yasukawa et al. J. Biol. Chem. 270, 25328-25331 (1995)) In this study we intended to assess whether thiol reductase or flavin reductase would both be equally effective at solubilizing the protein of interest; proteinase inhibitor 2. We attempted to create fusion protein constructs using the Novagen plasmid pET32a as a cloning vector since the pET32a contains thiol reductase. To compare reductase proteins, a flavin reductase gene was obtained for cloning. Due to shortage of time for this experiment, the constructs were not produced, however all of the fragments to be cloned and the control vector were produced. Additional time and resources would ensure that the constructs are produced and protein expression and analysis can be carried out to test the solubilizing properties of thiol and flavin reductases on proteinase inhibitor 2.

An obstacle when studying protein chemistry using proteins produced by expression systems is the aggregation and inactivation that can result in insoluble proteins. Creating a recombinant fusion protein with thiol reductase (Trx) (6) improves the characterization of the protein of interest. The solubility of the insoluble proteins were compared in native form and in the presence of thiol reductase (6). Many tested proteins that formed had insoluble aggregates had significantly increased solubility when expressed with Trx. Two proteins in particular: Myb and p53 tumour suppressor had significantly increased solubility (6). A commercially available vector, produced by Novagen (1) also utilizes the *trx* sequence to enhance the production of active protein products when produced in a bacterial system. It is unclear whether the effect arises from the fusion protein properties or the reductase activity. To test our explanations we intended to couple a different reductase protein with an insoluble protein such as proteinase inhibitor 2 (PI2) to determing if there is an increase in the solubility of PI2 as an uncoupled protein. Another reductase protein, flavin reductase (Fre), is hypothesized to have similar solublizing properties as the Trx with the PI2 protein. Designing a construct with a fusion protein of PI2 and Trx and comparing the protein solubility to another construct with PI2 coupled to Fre will determine whether both are effected and determine which reductase is more effective at producing an active and soluble protein product. Due to cloning obstacles and time limitations, the constructs containing PI2 were not obtained, however more research will surely allow the solubility of proposed constructs to be studied.

MATERIALS AND METHODS

The constructs were based on the Novagen plasmid pET32a (1) which contains *trx* and is available as a common cloning vector. The cloning strategy involved creating three separate constructs with the PI2 gene to compare solubility in the presence of no reductase, *trx*, and *fre*. The *trx* was to be removed from the pET32a to create a control plasmid into which PI2 was inserted. The two experimental plasmids were to be produced by cloning in the PI2 gene into the pET32a with *trx* and also into a pET32a plasmid with the *trx* removed and replaced with the *fre* gene. This would allow us to accurately compare the PI2 gene as a fusion with either one of the two reductases, or none in the case of the control construct. The PI2 gene was obtained in a plasmid known as pE32. The PI2 gene was cloned out of the plasmid using PCR amplification and forward and reverse primers were designed with engineered restriction sites to allow cloning later into the pET32a plasmid (Fig. 1). The fre product was obtained by PCR amplification out of pES1 plasmid using forward and reverse primers (Fig. 2). The primers were designed to change one stop codon to a glycine as well as change an EcoR1 restriction site to an Nde1 site in order to allow the insertion of the *fre* into the vector once *trx* was removed from the pET32a using flanking Nde1 restriction sites. After producing the three constructs containing PI2 and either no reductase, *trx*, or *fre*, the plasmids were to be transformed into an *E. coli* strain such as BL21 to express the proteins and allow characterization of solubility of PI2 each of the different constructs. PI2 expressed as a fusion with the reductase proteins are expected to be of greater solubility than the PI2 protein alone.

Preparation of Competent DH5α *E. coli* cells.

A 5 mL culture of DH5α *E. coli* cells was grown in Luria Bertani (LB) (4) broth at 37°C in a shaking incubator overnight. This starter culture was added to 500 mL of LB broth in the morning and grown for several hours at 37°C until the culture was harvested after approximately 3 hours. The cells harvested and then washed with 25 mL of 100mM $MgCl_2$ twice followed by one wash with 25 mL of 100 mM $CaCl_2$. The culture was then resuspended in 20 mL of 100 mM $CaCl_2$ containing 10% glycerol. Aliquots of 200 µL were stored at -80°C.

PI2 gene (GI: 41387698)

ccatgggcatggctgttcacaaggaagttaatttcgttgcttacctactaattgttctt
ggtaagattttcctttactccttttttttaaaaaaaataaaaaataaaaaatcttgctt
tatatatatatacacaagtagttttatattttccttttatattatatttgtttgtagga
ttattggtacttgtaagcgcgatggagcatgttgatgcgaaggcttgcactttagaatg
tggtaatcttgggtttgggatatgcccacgttcagaaggaagcccggaaaatcgcatat
gcaccaactgttgtgcaggttataaaggttgcaattattatagtgcaaatggggctttc
atttgcgaaggagaatctgacccaaaaaacccaaatgtttgcccccgaaattgtgatac
aaatattgcctattcaaagtgtccccgttcagaaggaaaatcgctaatttatcccaccg
gatgtaccacatgctgcacagggtacaagggttgctactatttcggtaaaaatggcaag
tttgtatgtgaaggagagagtgatgagcccaaggcaaatatgtaccctgcaatgtgagg
atccggc

Shaded: primers designed flanking PI2 gene
Black text: original PI2 gene obtained from GenBank
Underlined text: extra engineered nucleotides containing the restriction endonuclease Nco1 site in the forward primer and BamH1 in the reverse primer

FIG. 1 Primer design to clone the PI2 gene from pE32 plasmid. Primers were designed using GenBank sequence ID 41387698 for PI2 and the restriction sites of Nco1 and BamH1 were chosen due to the unique presence in the pET32a plasmid. The primers contain restriction enzyme sites to allow subsequent cloning into the pET32a with directional specificity by using enzymes cutting sticky unique ends. A PCR product of 597 bp is expected using these primers and template DNA.

fre Gene Cloning

TAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAG
AAGGAGATATACATATGACAACCTTAAGCTGTAAAGTGACCTCGGTAGAAGCTATCACG
GATACCGTATATCGTGTCCGCATCGTGCCAGACGCGGCCTTTTCTTTTCGTGCTGGTCA
GTATTTGATGGTAGTGATGGATGAGCGCGACAAACGTCCGTTCTCAATGGCTTCGACGC
CGGATGAAAAAGGGTTTATCGAGCTGCATATTGGCGCTTCTGAAATCAACCTTTACGCG
AAAGCAGTCATGGACCGCATCCTCAAAGATCATCAAATCGTGGTCGACATTCCCCACGG
AGAAGCGTGGCTGCGCGATGATGAAGAGCGTCCGATGATTTTGATTGCGGGCGCCACCG
GGTTCTCTTATGCCCGCTCGATTTTGCTGACAGCGTTGGCGCGTAACCCAAACCGTGAT
ATCACCATTTACTGGGGCGGGCGTGAAGAGCAGCATCTGTATGATCTCTGCGAGCTTGA
GGCGCTTTCGTTGAAGCATCCTGGTCTGCAAGTGGTGCCGGTGGTTGAACAACCGGAAG
CGGGCTGGCGTGGGCGTACTGGCACCGTGTTAACGGCGGTATTGCAGGATCACGGTACG
CTGGCAGAGCATGATATCTATATTGCCGGACGTTTTGAGATGGCGAAAATTGCCCGCGA
TCTGTTTTGCAGTGAGCGTAATGCGCGGGAAGATCGCCTGTTTGGCGATGCGTTTGCAT
TTATCTGAGATATAAAAAAACCC

Shaded: primers designed to amplify *fre* gene
Black text: *fre* gene within the pES1 plasmid
Underlined text: induced mutation sites to change stop codon at the end of the *fre* gene to a glycine codon and to engineer a restriction site for NdeI

FIG. 2 Primer design to amplify flavin oxidoreductase (fre) gene out of the plasmid pES1. The yellow highlighted region indicates the primer-binding site of the start of the fre gene. The reverse primer contains mutations to replace the stop codon with a glycine to create a fusion protein with PI2, and introduce an Nde1 restriction enzyme site. The PCR product is expected to be 788 bp.

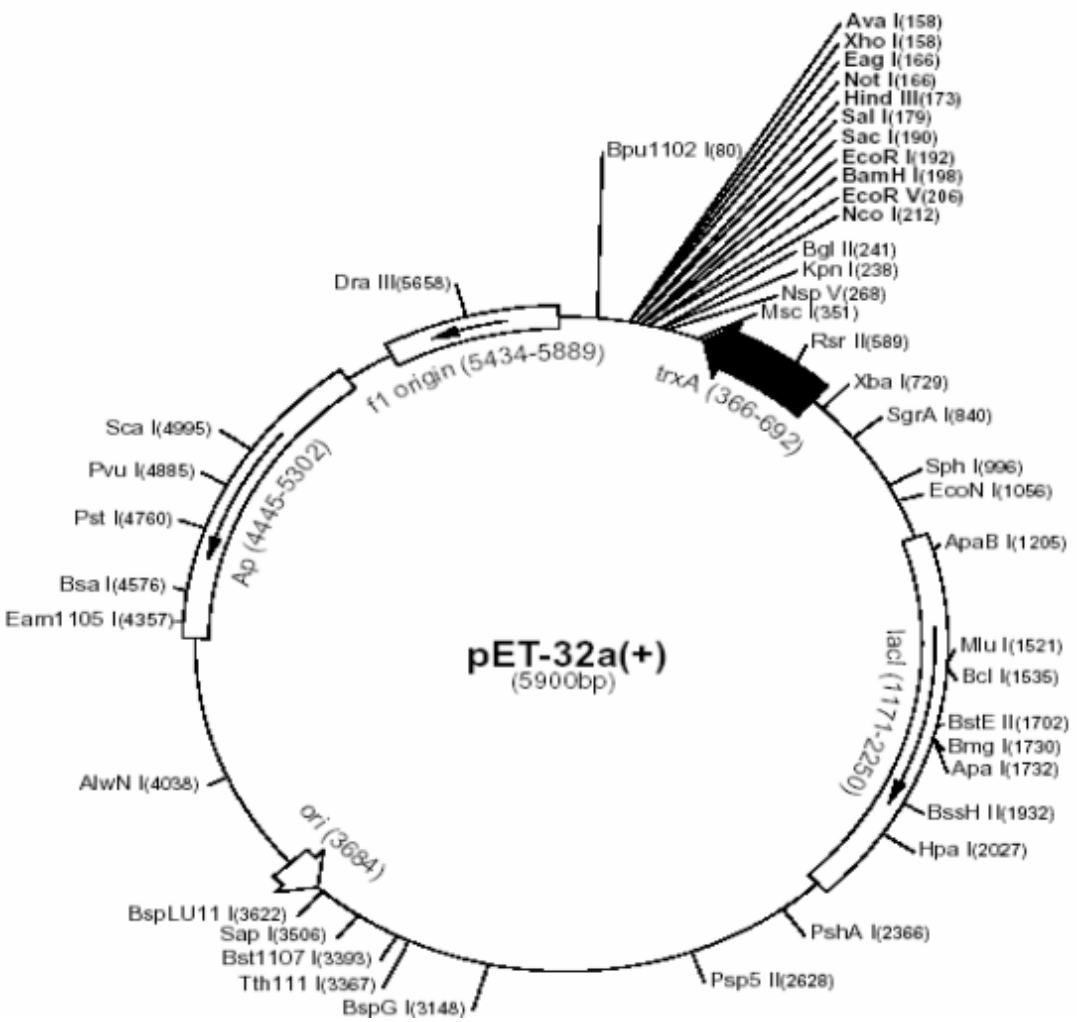

FIG. 3 Novagen pET32a vector (1). The Nco1 and BamH1 restriction sites are present in the multiple cloning site of the plasmid where the PI2 will be inserted. The vector contains the trxA sequence flanked by two Nde1 restriction sites. These Nde1 restriction sites will allow trx to be removed and fre inserted substituted to generate the constructs containing different reductase sequences with PI2 as a fusion protein.

Amplification of the PI2 gene from the pE32 plasmid.
As no plasmid map was available for the plasmid containing PI2, PCR amplification was done using GenBank sequence for PI2 as outlined in Fig. 1. Forward and reverse primers were designed to anneal to the PI2 sequence, and have overhangs containing unique restriction sites to allow for insertion into the vector later. The forward primer contained a restriction site for Nco1 and the reverse for BamH1. These restriction sites were so chosen because there was only one copy of each sequence present in the pET32a vector (Fig. 3).

These sites allow the plasmid to be cut open within the multiple cloning sites so the PI2 fragment can be inserted in the correct orientation within the plasmid. Primers were ordered from the Nucleic Acid and Protein Sequencing facility at the University of British Columbia (NAPS) and rehydrated at a concentration of 20µM each. PCR was performed on uncut pE32 plasmid using Platinum Pfx DNA polymerase from Invitrogen Life Science (2). In all cases the amplification reactions were performed in total volumes of 30 uL containing 1 uL of extracted DNA, and final concentrations of: 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each of four deoxynucleotide triphosphates, 0.5 uM of each primer, and 1 unit Pfx polymerase. PCR conditions were based on standard PCR protocols. PCR cycles began with an initial denaturation at 94°C for 3 min followed by 40 cycles of denature at 94°C for 30 s, anneal at a gradient from 45°C to 65°C for 1 min, and extend at 72°C for 90 s followed by a final anneal at 72°C for 10 min. The gradient was used to attempt to obtain optimal PCR products from the reactions.

Amplification of the *fre* gene from pES1 plasmid.
Forward and reverse primers designed to flank the *fre* gene in the pES1 plasmid. Primer design and annealing sites are seen in Fig. 2. The forward primer anneals to start of the *fre* sequence and the reverse primer contains mutations to create a suitable sequence for cloning and fusion protein production. The reverse primer mutates a stop codon into a glycine to allow a fusion protein to be produced later with PI2. An Nde1 site was introduced in order to clone the *fre* fragment into the pET32a plasmid once the *trx* fragment was cut out using Nde1 sites. The *fre* gene will then insert where the *trx* was removed. Primers were ordered from NAPS and rehydrated to 20µM concentrations each. Each amplification reaction had final concentrations exactly as those in the PI2 reactions, except that pES1 plasmid was used as the template instead of pE32 plasmid. PCR began with an initial denaturation at 94°C for 3 min followed by 40

cycles of denature at 94°C for 30 s, anneal at 54°C for 1 min, and extend at 72°C for 90 s followed by a final anneal at 72°C for 10 min.

Cloning of *fre* gene into pET32aΔtrx. pET32aΔtrx vector was provided by Aileen Kartono. The PCR amplified *fre* fragment from pES1 plasmid was prepared for cloning by purification using Qiaquick PCR Purification Kit (5) to remove nucleotides and other reaction components. The *fre* fragment was digested using Nde1 restriction enzyme to generate a sticky ended fragment. The reaction was heat inactivated and then used to set up ligations into pET32aΔtrx vector already linearized by the removal of *trx*. Several ligation reactions were set up as indicated in Table 1 (2). Many different conditions were tested to better the chances of obtaining a ligated product. Once allowed to ligate, each ligation reaction was transformed into chemically competent DH5α cells.

TABLE 1. Conditions for the ligation reactions of *fre* into the pET32aΔtrx.

Reagents	Reaction				
	1	2	3	4	5
Insert (cut *fre*) (uL)	3	6	9	9	9
Vector (pET32a Δtrx) (uL)	1	2	3	3	3
Buffer (uL)	4	4	4	4	4
Ligase (uL)	2	2	2	1	1
dH_2O (uL)	10	6	2	3	3
Total volume (uL)	20	20	20	20	20
Ligation conditions (temperature and time)	15°C 24h	15°C 24h	15°C 24h	25°C 20 min	25°C 16h

Transformation of DH5α cells with ligation reaction. 2 µl of each ligation reaction in Table 1 was added to 100µl of chemically competent cells thawed on ice. The mixture was allowed to equilibrate on ice for 10 minutes and then incubated at 42°C for 90 seconds. After another 10 minutes on ice, 1 mL of SOC (4) broth was added. The cultures were incubated at 37°C in a shaking water bath for 3 hours. 200 µl of each of the cultures was then plated on an LB agar plate (4) containing 50µg/mL ampicillin to select for bacteria containing the plasmid of interest. The plates were incubated overnight at 37°C.

Insertion of PI2 gene PCR fragment into pET32a and pET32aΔtrx vector. PI2 PCR product purified from agarose gel after analyzing by gel using Qiagen Gel Extraction Kit (5). Used 600 uL of buffer QG for solubilizing agarose and elution in 2 x 20 uL buffer EB. Double digested PI2 purified fragment, pET32a vector, and pet32aΔTrx vector each with both restriction enzymes BamH1 and Nco1. After digestion, reactions were heat inactivated and ligations set up as in Table 2 and incubated at 4°C overnight (2). The reactions were then transformed using chemically competent DH5α cells and plated on LB agar plates (4) containing 50 µg/mL ampicillin.

RESULTS

Amplification of PI2 gene from pE32 plasmid. The agarose gel of the products of the gradient PCR to amplify PI2 showed many bands and background noise. There were many bands amplified as seen in Fig. 4 and there is some smearing seen in the lanes, especially in lane 2. This smearing could be the result of degradation of some of the DNA products in the PCR reaction as well as overloading of the agarose gel lanes. The expected size of the gene product is 597bp and this size fragment is seen in lanes 8 through 12. These lanes correspond to the annealing gradient temperatures of 56.5°C, 59.3°C, 61.8°C, 63.8°C, and 65.0°C. These higher annealing temperatures are the preferred temperatures for amplifying the PI2 gene and it appears that lanes 10 and 11 have the brightest bands of the five positive lanes for the PI2 fragment.

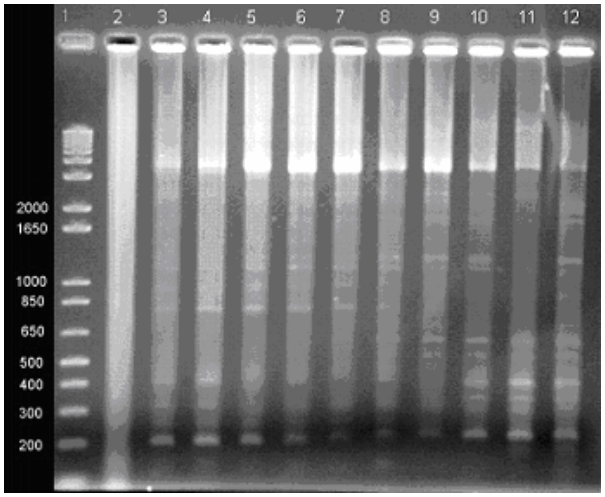

FIG. 4 Gel image of PI2 amplification from pE32 in gradient PCR. A 1% agarose gel image of the gradient PCR. Lane 1 contains 1ug of 1kB plus ladder (2). Lanes 2 to 12 contain PCR reaction samples ranging in gradient temperature from 45°C to 65°C. The 597 bp fragment from lanes 8 to 12 were excised from the gel for gel purification to isolate the correct fragment. Lanes 8 to 12 correspond to temperatures of 56.5°C, 59.3°C, 61.8°C, 63.8°C, and 65.0°C.

Amplification of *fre* gene from pES1 plasmid. The amplification of the *fre* gene was successful and a PCR product of 788 bp. The agarose gel image of the PCR is not shown here but was identified as the correct length visually. The *fre* fragment was purified from the PCR reaction mixture using Qiagen PCR Purification Kit (5) before being used in any further cloning.

Cloning of *fre* gene into pET32aΔtrx vector. After incubation, no colonies were seen on any of the plates. The various ligation reaction conditions were all unsuccessful in producing a vector containing *fre* in place of the *trx*. A control of plasmid pES1 also containing the same antibiotic resistance was used as a transformation control, which resulted in the growth of numerous colonies. The growth of the control indicates that the competent cells were viable but the ligations were not successful.

Insertion of PI2 PCR fragment into pET32a and pET32aΔtrx vector. After incubation, no colonies were seen for either ligation reaction into the vectors. A control transformation was also prepared, which

TABLE 2. Conditions for the ligation reaction of pET32aΔtrx and pet32a with PI2.

Reagent	Volume (uL)	
	Ligation reaction 1	Ligation reaction 2
5x ligase buffer	4	4
Ligase	1	1
PI2 DNA	10	10
pet32aΔtrx DNA	5	-
pET32a DNA	-	5
Total reaction	20	20
Conditions: Overnight at 4°C		

again had numerous colonies on the agar. The ligation reactions were unsuccessful at producing a colony containing the PI2 gene.

DISCUSSION

Amplification of *fre* gene from pES1 plasmid. We obtained a fragment corresponding to the correct size of 788 bp which is the expected product of *fre*. The amplification of the product indicates that the primers and the PCR conditions were optimal for this gene and the yield was also high when analyzed on agarose gel. The fact that the PCR was successful indicates that the engineered mutations needed to clone the *fre* into the pET32aΔtrx vector were also successful. The primers were designed to ensure that when *fre* was inserted into the vector, then the protein produced would be a complete fusion protein of the protein of the PI2 and *fre*. While analysis by agarose gel to assess the size of the fragment generated was sufficient identification for our purposes, a more reliable and accurate measure of the sequence of the product would be to sequence it to ensure that all the nucleotides are correct if the construct behaved improperly during protein expression experiments. With a correct sequence during the cloning stages, protein expression at later stages can be optimized when the nucleotide sequence codes for the correct proteins. Checking the sequence of the completed construct when it is obtained would assure that the fusion protein produced is indeed what is expected.

Cloning of *fre* gene into pET32aΔTrx vector. We planned on cloning the Fre gene into the vector using the same restriction sites that remove the trx from the pET32a. These restriction sites for Nde1 were engineered onto *fre* for the purpose of cloning it into the pET32aΔtrx vector. As the *fre* would insert directly in frame with the removal of the *trx*, a complete sequence was expected with no errors of frameshift or lack of protein production at a later stage. After many attempts of cloning the *fre* into pET32aΔtrx there was no success at obtaining a construct. A variety of conditions were utilized as well as using fresh ligase enzyme and buffer, all to no success. This stage of cloning would need to be re-examined for possible problems inhibiting the ligation reactions. Such problems could be the Nde1 enzyme cutting the DNA but also creating an inhibition effect and blocking the ligation sites. Heat treatment of the DNA to be ligated was attempted prior to doing the ligations in order to release any protein that may be associating with the DNA, however this did not help to obtain a successful construct. The DNA fragments of *fre* and pET32aΔtrx were heated to 95°C and very slowly cooled to 25°C. This step should have denatured any proteins present but still allowed the DNA to reanneal. As the Nde1 sites were already engineered and are a convenient cloning site, another way to obtain the same cut fragments for ligation would be to use another restriction enzyme that cuts at the same site as Nde1 but has different properties that may not inhibit subsequent ligations. Such an enzyme that cuts at the same site is an isoschizomer of Nde1 and an example of such a commercially available enzyme is BfuC1. This is a possible alternate method to obtaining the correct constructs.

Insertion of PI2 PCR fragment into pET32a and pET32aΔTrx vector. As time was a factor in carrying out this experiment, only one attempt was made to clone the PI2 gene sequence into the two available vectors pET32a and pET32aΔtrx. The digestion of the vectors and the PI2 sequence in preparation for cloning likely was successful at creating ends that would anneal given the appropriate conditions. The ligation may have been more successful at a higher temperature for a shorter period of time, for example 15°C for a few hours instead of the longer incubation that was attempted. It is predicted that the ligation would be successful on subsequent attempts because the restriction sites are sticky ends and since each end of

the gene has a different site, the fragment will insert in the correct direction to allow for protein expression later. The PI2 fragment primers were designed to keep the protein in frame with the vector to allow successful production of a fusion protein with either *trx* or *fre*.

FURTHER RESEARCH

The pET32aΔTrx vector construct containing *fre* must be obtained, and with further experimentation the construct can be produced successfully. The PI2 gene must be successfully inserted into all three vectors: pET32a, pET32aΔtrx, and pET32aΔtrx+fre. Any resulting colonies from these ligation reactions and transformation would need to be screened to check for the correct insert. If required, the plasmids could be sequenced to verify the identity prior to further experimentation. Once the positive constructs are available, the plasmid can be transformed into the strain of *E. coli* BL21 for protein expression analysis. Appropriate protein isolation techniques to isolate the fusion proteins would need to be designed and carried out to analyse the amount of soluble protein present compared to the amounts of aggregated insoluble proteins.

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