Making a Construct Containing the IF3 Gene in a pET16b Plasmid

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Abstract

My research included inserting the IF3 gene from a pGEM-3Z plasmid into a pET16b plasmid that would later be transformed into an *E. coli* cell. The DNA for initiation factor 3 (IF3) was first amplified to significant amounts by PCR (polymerase chain reaction), then the PCR product and the pET16b plasmid were cut with restriction enzymes. This was followed by ligation (sticking the insert into the plasmid) and transformation (the uptake of the plasmid into *E. coli* cells). The *E. coli* host cell can then be induced to express the target protein through the T7 RNA polymerase gene. The pET16b plasmid was chosen because it contains a His-tag that allows the expressed protein to bind to an affinity column. The IF3 protein is essential in translation as it binds to the 30S ribosomal subunit to dissociate it from the 70S particle, allowing initiation to take place.

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Introduction

Translation involves deciphering nucleotide triplets on the messenger RNA (mRNA) into chains of amino acids, in other words, a protein. To achieve the final product of a protein a go-between is needed to attach to the codons (nucleotide triplets). [1] The “adaptor molecules” are the transfer RNA (tRNA) which are like “flashcards” in that one side contains the anti-codon needed to match the codon, whilst the other side holds an amino acid specifically coded for that nucleotide triplet.[2]

However, before the tRNA can attach to the mRNA at specific sites, the mRNA first has to be attached to the 30S subunit. But once translation occurs and the protein and mRNA are freed, the 30S subunit remains bound to the 50S subunit maintaining the 70S ribosome. This is where the IF3 protein comes into play. The IF3 and IF1 bind to the 30S subunit, freeing it from the 50S subunit, allowing another protein to be expressed. [3]

Initiation factor IF3 is a protein with 2 domains-the N domain and the C domain with a flexible linker joining the two. According to recent research, it is the IF3 C-domain that carries out all the functions of the IF3 itself when added in sufficient amounts to compensate for its reduced affinity to the 30S subunits. [4] Functions of IF3 include dissociating the 70S ribosome, releasing the 30S subunit and ensuring fidelity to the initiation sites. By ensuring fidelity, it actually binds to the A site on the 70S ribosome, forcing the fMet-tRNA to bind to the P site immediately, instead of binding to the A site as all other tRNAs will do, following the start codon of fMet. [5]

In my experiments, I worked specifically with the IF3 gene initially from the bacteria *Escherichia coli* (*E.coli)* in a pGEM-3Z plasmid. The aim of the experiment was to amplify the gene from the pGEM-3Z by the polymerase chain reaction (PCR) and then to put the amplified gene into a pET16b plasmid containing a His-tag codon. The His-tag is a chain of six Histadines in front of the Nde I site on the pET16b that allows for easy purification through immobilized metal affinity chromatography (IMAC) once the gene has been expressed. But before the protein can be purified, the plasmid containing the IF3 gene first has to be transformed into an *E.coli* cell so the IF3 protein can be expressed and then purified. The recombinant plasmid was transformed into a XL1B *E.coli* cell for propagation. Later on, the protein will then be purified through over expression in BL21 *E.coli* cells by IMAC.

The main aim of my research was to make a construct containing this IF3 gene from *E.coli* with a His-tag codon in front and then transform it into XL1B *E.coli* cells for expressing. The expressed IF3 would then be made part of an initiation complex consisting of *E.coli* ribosomes, the *E.coli* IF3 and the yeast mitochondrial IF2 as part of an ongoing research project here at the university covered by the Appling and Hardesty labs.

Procedure

Initially appropriate forward and reverse primers were designed for the PCR reaction. The forward primer was required to have a CATATG sequence of nucleotides because of the Nde I site whilst the reverse primer had to have a Bam HI site which is defined by the GGATCC sequence of nucleotides. The remaining 17 or so nucleotides had to match the sequence of the IF3 gene as closely as possible to ensure a better chance for the primer to form more hydrogen bonds, making the primer and template bond stronger which meant it would not dissociate so easily, giving it a better chance to be extended by the *Taq* polymerase. The designs of the primers were such that the forward primer so named IF3 forward fit almost perfectly to the template but the reverse primer had more alterations in its sequence of nucleotides.

The first polymerase chain reaction was carried out on the IF3 pGEM-3Z sample to amplify the IF3 gene to significant amounts to deal with. Several trials had to be carried out to finally get the correct ratio of materials. The unsuccessful PCR trials #1, #2, and #3 probably had too little magnesium chloride, which could have meant that there were not enough magnesium ions available for the dNTP. Also we speculated that it was the primers that were not binding. We then experimented with different primers, namely the IF3 forward and IF3 reverse that we designed, as well as the T7 forward and M13 reverse which were originally used with the plasmids. The M13 and IF3 forward combination proved successful with added magnesium.

In the first PCR trial there was 11 µl of PCR mix, which contained 2.5µl of 10X PCR buffer, 1.8µl of 50mM MgCl2, 2.0µl of IF3 forward primer. 2.0µl of IF3 reverse primer, 0.15µl of Taq polymerase and, 2.5µl of dNTP which is a mixture of 2.5mM dATP, dGTP, dTTP, and dCTP. These solutions made up the first PCR mix. I prepared 3 tubes, one control, one with 2µl of the IF3 pGEM-3Z solution and one with 4µl of the IF3 pGEM-3Z solution. The total reaction mix was 25µl for each tube. Then, mineral oil was added to prevent the mixture from evaporating. The mixture was run under file 39 on the PCR machine which has a denaturing temperature of 94°C, an annealing temperature of 55°C and an extension temperature of 72°C. The PCR was allowed to run overnight which meant once it finished running the 26-30 standard cycles the samples were kept chilled at a temperature of 4° C in the machine.

Two more PCR trials were carried out this time trying all four primers, the IF3 forward, the IF3 reverse, the M13 reverse, and T7 forward with negative results. Finally, by changing the concentration of the Magnesium Chloride by a drastic amount and changing the file on the PCR machine to file number 10 which has an annealing temperature of 50°C and using more IF3 pGEM-3Z. The final positive result mixture was 4.2µl of PCR mix which contained 2µl of 10X PCR buffer, 2µl of dNTP, and 0.2µl of *Taq* polymerase. To that was added 6µl of the IF3 pGEM-3Z, 2µl of the M13 reverse primer, 2µl of the 1F3 forward primer and 5µl of Magnesium chloride 50mM. A mistake was made here as I did not dilute the 50mM MgCl2 to 10mM as I was supposed to, but the large amount of Mg(2+) did not seem to matter. Another change was that we used a newer *Taq* polymerase obtained from Dr. Appling's lab. However, using we used the old

Taq polymerase in the next larger PCR trial to obtain more material and that worked as well, so it must not have been the *Taq* that had been causing the error.

As soon as the successful PCR results were obtained, those ratios were closely replicated and increased to obtain a larger result that would then be used for the ligation. The larger ratios used were 10µl of 10X PCR buffer, 20µl of diluted IF3 pGEM-3Z, 1.5µl of *Taq* polymerase, 8µl of 1F3 forward primer, 8µl of M13 reverse primer, 10µl of dNTP, 5µl of MgCl2, and 37.5µl of autoclaved water.

The successful PCR product then needs to be purified with a QIAQuick PCR purification kit. 5X the volume of Buffer PB is added to 1X the volume of the PCR sample and mixed. Then a QIAquick spin column is placed in a 2mL collection tube. Then the sample is applied and centrifuged for 30-60 seconds in a mini centrifuge to bind the DNA to the column. The flow through is discarded and the column is placed back into the same tube. Then 0.75mL of Buffer PE with ethanol is added to the column and centrifuged in the mini-centrifuge for 30-60 seconds. The flow through is discarded again, but then the column is centrifuged at a higher speed for an extra minute to get rid of excess ethanol. The column is then placed in a clean 1.5mL tube and eluted with 50µl of Buffer EB applied to the center of the membrane and allowed to sit for 5 minutes before centrifuging for 1 minute at a high speed.

Then an enzyme digest was run to cut the PCR sample of the IF3 gene and the pET16b vector to prepare them for ligation. In the first tube, I used 50µl of the PCR product, 6µl of the 10X Multicore buffer approximately 0.6µl of the 10X BSA, 1µl of Nde I, and 1µl of Hind III. In the second tube, there was 30µl of pET16b plasmid, 4µl of 10X Multicore buffer, 4µl of 1X BSA, 1µl of Nde I, and 1µl of Hind III. We had to use the Multicore buffer because all other buffers provided did not work well with the combination of Nde I and Hind III. The efficiency of the Multicore with Nde I was 25-50% whilst the efficiency of Multicore with Hind III was 50-75%. However, the pET16b product of the enzyme digest was lost during the loading of a low-melting point gel, so I had to re-run the experiment, which meant I got more PCR product than plasmid.

After the enzyme digest was run and the samples put through a gel, I had to perform a gel extraction to extract the plasmid and IF3 gene from the gel. Using the QIAGEN gel extraction kit, I first cut the selected DNA fragment using a scalpel and weighed the sample. Then 3X volume of the gel of Buffer QG was added and the mixture was incubated for 10 minutes at 50°C. The sample is then removed and vortexed. Then 1X gel volume of isopropanol is added. The mixture is then applied to a column and centrifuged in a mini-centrifuge for 1 minute. The flow through is discarded and 0.5mL of Buffer QG is added to the column and centrifuged in a mini-centrifuge for one minute. To wash the product, 0.75mL of Buffer PE with ethanol is added to the column and centrifuged for 1 minute in a mini-centrifuge. The flow through is discarded and the column is again centrifuged for one minute but in a higher centrifuge machine. The column is placed in a clean 1.5mL tube and to elute the DNA, 50µl of Buffer EB is added to the center of the membrane, allowed to stand for about 5 minutes and then centrifuged in the higher centrifuge machine for 1 minute.

Once the purified plasmid and insert IF3 gene have been obtained through gel purification, the two can then be bonded covalently by ligation. Using a ligation kit, a ligation mixture is assembled in a micro tube consisting of 2.0µl of 10X Fast-Link Ligation buffer, 2.0µl of 10mM ATP, 9µl of vector DNA, 6µl of insert DNA, and 1.5µl of Fast Link DNA ligase. The reaction is incubated for anywhere from 5 minutes to overnight at room temperature. Then to inactivate the DNA ligase, the reaction is transferred to a 70°C water bath for 15 minutes. The tube is spun briefly in a micro-centrifuge and the plasmid and insert should be ligated.

After ligation, transformation into competent XL1B *E.coli* cells are performed on the recombinant plasmids. This was done by first placing 50µl of competent *E.coli* cells on ice. Then 2 agar plates containing LB and ampicillin were set out. 3µl from the ligation mix is put into a sterile 1.5mL micro tube. The 50µl of competent cells are added to the 1.5mL micro tube and put on ice for 20 minutes. Then the tube is heat-shocked at 41°C-42°C in a 70°C water bath for 1 minute. The tube must not be shaken. Then the tubes are put back on ice and 100µl of sterile LB is added to the tube. It is then incubated at 37°C for one hour with shaking. After an hour of incubation, 100µl is taken from the tube and spread onto a labeled LB-amp plate and the other 50µl is spread onto a separate labeled LB-amp plate. The plates are left on the bench for about an hour to let the liquid soak in and are placed in a 37°C oven overnight to allow the colonies to grow.

After transformation, it is required to test if the resulting colonies from the agar plates actually contain the plasmid. Thus, another PCR test needed to be run with several of the colonies with the T7 promoter, and the IF3 reverse promoter that stops after the 103rd amino acid. The colonies need to be boiled before being added to the PCR mix. The colonies are boiled in 30µl autoclaved water for five minutes. Then 7.5µl of the boiled colonies are added to 7.5µl PCR mix which contains 2.4µl of 10X PCR buffer, 1.8µl of 50mM MgCl2, 2.0µl IF3 reverse “103”, 2.0µl T7 promoter, 2.5µl of dNTP, and 0.15µl of *Taq* polymerase.

After ensuring the plasmid and insert is correct, the cells need to be grown to a significant amount, so they are put into a culture. The positive colonies are selected. Then in a test tube I put 3mL of LB and 1.5µl of ampicillin. The colonies number 1 and number 5 (refer to appendix I) are scraped and swirled in the LB-amp mix. They are then put in a cell grower at 37°C that swirls the mix encouraging cell growth and multiplication. Later after several hours, the mixture is diluted to 5 ml by adding another 2µl of LB to the mix and leaving the cells to grow in the 37°C environment.

When the cells observed look turbid, they are removed, and a plasmid prep needs to be run on the positive colonies. The cells are first re-suspended in 250µl of Buffer P1 and transferred to a microfuge tube. Then 250µl of Buffer P2 is added and the tube is inverted 4-6 times to mix. 350µl of Buffer N3 and the tube is inverted immediately 4-6 times. The mixture is then centrifuged on high for 10 minutes. Then the supernatants from the centrifugation are placed into a QIAprep spin column in a 2mL collection tube. The

column is then centrifuged in the micro-centrifuge for 30-60 seconds and the flow through is discarded. Then the DNA is washed with 0.5mL of Buffer PB and centrifuged for 30-60 seconds in the micro-centrifuge and the flow through discarded. Then the column is washed by adding 0.75mL of Buffer PE with ethanol and centrifuged for 30-60 seconds in the micro-centrifuge. Then the flow through is discarded and the column is centrifuged once again on a higher speed for 1 minute to remove residual ethanol. Then the column is placed in a clean microfuge tube and the DNA is eluted by adding 50μl of Buffer EB to the center of the membrane, and sat on the counter for about 5 minutes, and then centrifuged at a high speed for one minute.

Following the plasmid prep, to obtain the purified protein, IMAC needs to be carried out. The procedure being first thawing the cells. Then 10mL of 1X Binding buffer is added to the nickel column. Then add 1/75 volume of the 75mM PMSF. The mixture is mixed by pipetting the mixture up and down several times. Then, the cells are broken apart with a sonificator for approximately two 15-second sessions. Then the mixture is centrifugated in an ultra-high refrigerated centrifugator for about 20 minutes. The supernatant is kept whilst the solid and cell debris is thrown out. Then the supernatant from the centrifugation is run through the column not allowing the top of the liquid to go below the Nickel charged cloudy blue part of the column. The liquid run through the column is kept. When the supernatant is just about to go past down the column, the column is washed with 10mL or approximately 3.5X the volume of the column of 1X Binding buffer. Then the column is washed with approximately 3.5X the volume of the column of 1X Wash buffer. 15 sterile micro tubes are prepared numbered 1-15. Finally the column is eluted by filling the column full with 1X Elute buffer and allowing approximately 6-7 drops into each sterile micro tube labeled one to fifteen. The order it proceeds in is such that 6 drops are allowed into number tube number one, then the next 6 into tube number 2, the next 6 into number 3, and so on until all fifteen are filled at which point the column is closed.

The purified protein is then spotted on a protein identifier special paper that shows the presence of proteins in the each of the solutions. A remaining spot of blue dye confirms the presence of protein even after the paper has been washed in a dye remover solution (Appendix J). The solutions that test positive on this paper are then run on a protein gel to check their molecular weights to ensure that the protein purified is the correct protein. Refer to Appendix K for example of protein IF2 on a protein gel.

Results and Conclusions

There were three unsuccessful PCR trials before the fourth trial that gave a positive result. There were too many variables to keep track of to be able to tell without a doubt what caused those three unsuccessful trials. However, it is clear that more magnesium was needed as when more was added, both trials succeeded. However, in the last PCR dealing with the larger quantity, one test tube gave a positive result while the other gave no result, even though both contained exactly the same amount of each material and were exact replicas of each other (Appendix E). Thus it can only be concluded that PCR can be somewhat unreliable depending upon many variables that can be subject to change. Perhaps the primers we designed were not particularly appropriate giving about a 50%

chance of binding and extension, or the programs chosen were not particularly appropriate for the given primers, as their melting points were around 57°C.

Following the successful PCR process, enzyme digests and ligation are the next steps. However, the enzymes Nde I and Hind III in the Multicore buffer had relatively low efficiencies. That could have been the cause of incomplete cutting which could mean that the plasmid was not cut properly, or cut only with the Nde I or only the Hind III and when ligated merely joined back together to form the plasmid with no insert inside. This would probably the majority of the colonies grown on agar plates as evidenced in Appendix H. The enzyme digest with the colonies chosen shows a tiny insert only about 200 base pairs long which means that the plasmid inserted into the cell did not contain the IF3 gene which is approximately 540 base pairs long, and about 700 base pairs in length when added with the primers.

Furthermore transformation is not a process that guarantees every single plasmid containing the IF3 gene insert is transformed into an *E.coli* host cell. As it is mostly impossible to tell how many plasmids were properly ligated and actually contained the inserts instead of just being stuck together again as explained in the last paragraph, the chances of a colony containing the correct plasmid with the correct insert within are rather slim, or in this case 1 out of 10 (Refer to Appendix G).

The 10 colonies chosen were run through the PCR process to check their insert sizes and were plated on a fresh agar plate. Only number 10 gave a positive result which would not have been a problem to just remove the growing colony and re-plate it and grow a cell culture from it (Appendix G). But there were several runs on the plate as the plate used was very fresh having just arrived that day, causing the positive and negative samples to mix in three large smears flowing vertically down the plate. This was another source of error that could have been a possible explanation for the results in Appendix H in which no plasmids seemed to contain the IF3 gene.

In reference to Appendix G, there was only one positive, with nine negative. Therefore when the samples on the plates ran into one another because of the slickness of the fresh agar plates, the chance of getting a correct colony that contained the desired colony #10 was slim. But on that doomed plate there remained a small spot of what appeared like colony 10, and so by blind trial and error we tried scraping that colony and plating it. When PCR was run on it, the results proved most encouraging with ½ positive results. However, (refer to Appendix I) sample number 6 seems too large. There is no indication so far of what that insert could be, whether it is some impurity or actually the IF3 gene itself with something else attached to it. But samples 1 and 5 are exactly the right size as the PCR primers used were the initial IF3 reverse we designed, which adds approximately 100 plus base pairs to the original 540 base pairs of the IF3 gene. The T7 promoter used also extends the length of the insert to the length of about 750 base pairs, which is almost exactly where the insert is, thus it can be concluded that sample 1 and 5 contain the IF3 gene and should be carefully cultivated to gain the IF3 proteins.

The successful XL1B cells can now be used to gain the proteins. The next step will be the purification of the IF3 protein through over expression in BL-21 host cells that contain a T7 RNA polymerase which is crucial as a part of expressing the protein. The proteins will be purified by immobilized metal affinity chromatography, with the metal being nickel. The proteins will then be tested on the special protein presence paper, and the positives will be run on a protein gel to ensure that the protein obtained is the correct one relative to the true molecular weight of the IF3 protein. The purified IF3 will be needed to form as part of an initiation complex on the *E.coli* ribosomes but with yeast mitochondrial IF2 instead as part of an ongoing research project on yeast mitochondrial functions and proteins, namely the mitochondrial IF2.

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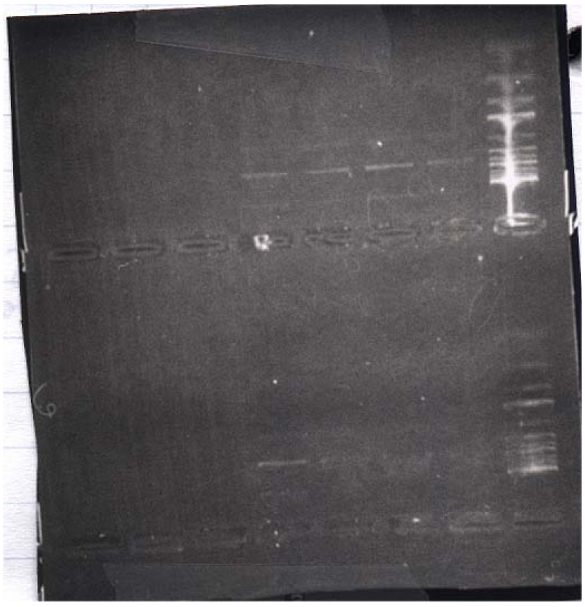
Appendix A

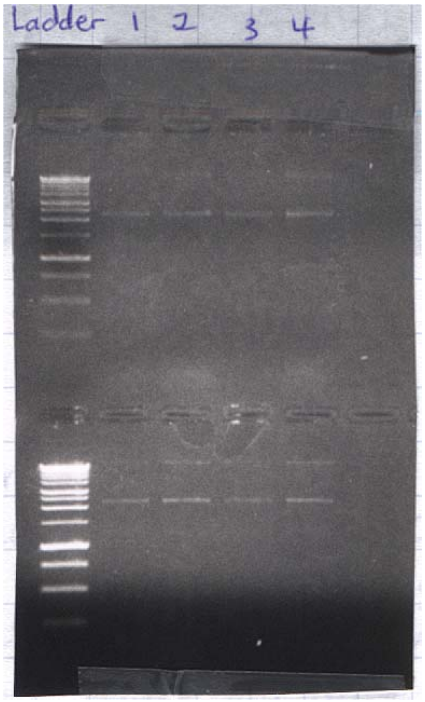
PCR Trial #1 Results on Gel.

Appendix B.



PCR Trial #2 on Gel.

Appendix C.



PCR Trial #3 on Gel.

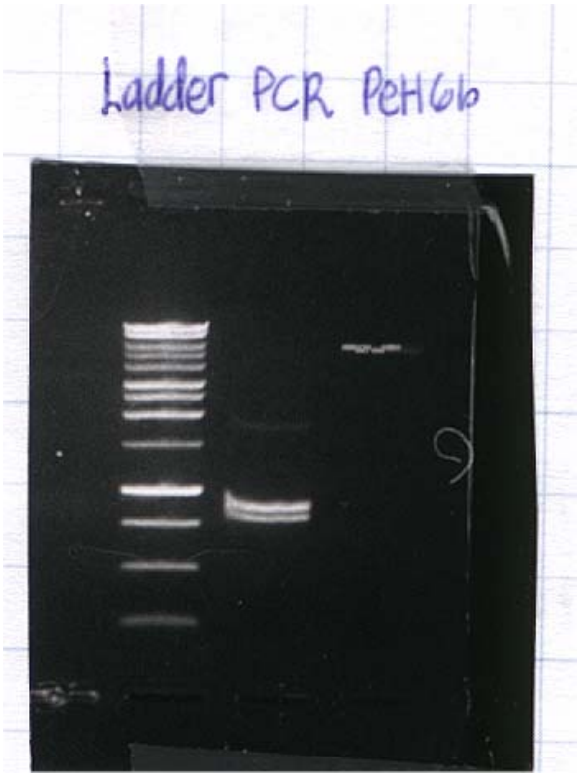
Appendix D.

PCR Trial #4 on Gel with positive result #6.

Appendix E.

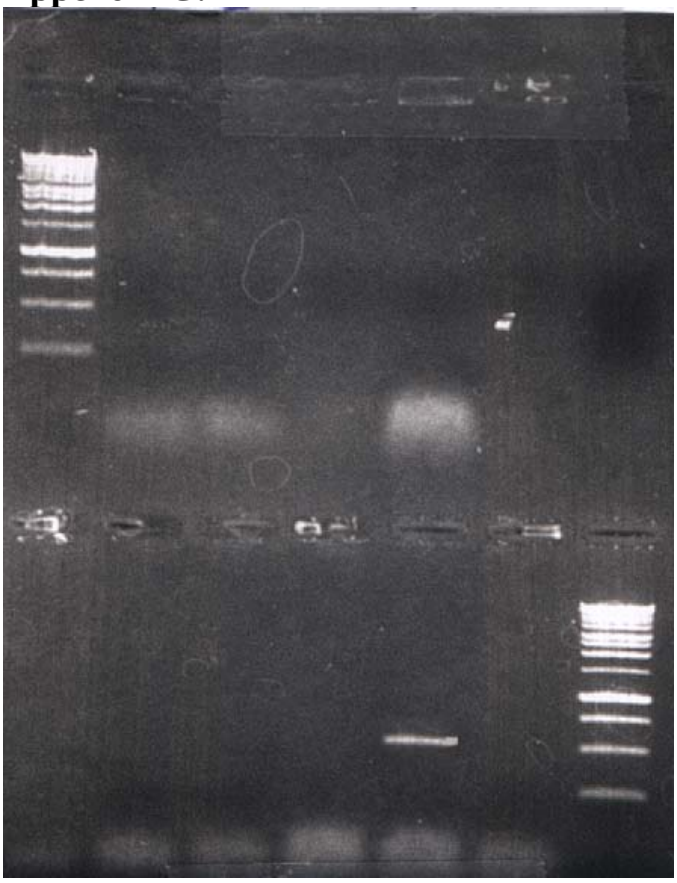
PCR Larger quantity on Gel

Appendix F.



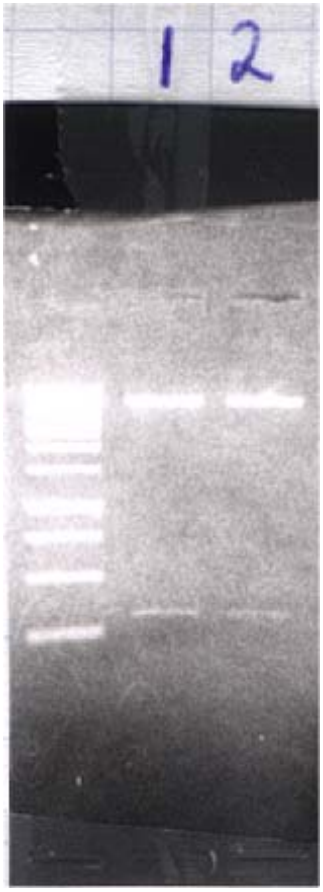
Enzyme Digest on PCR product and pET16b vector.

Appendix G.



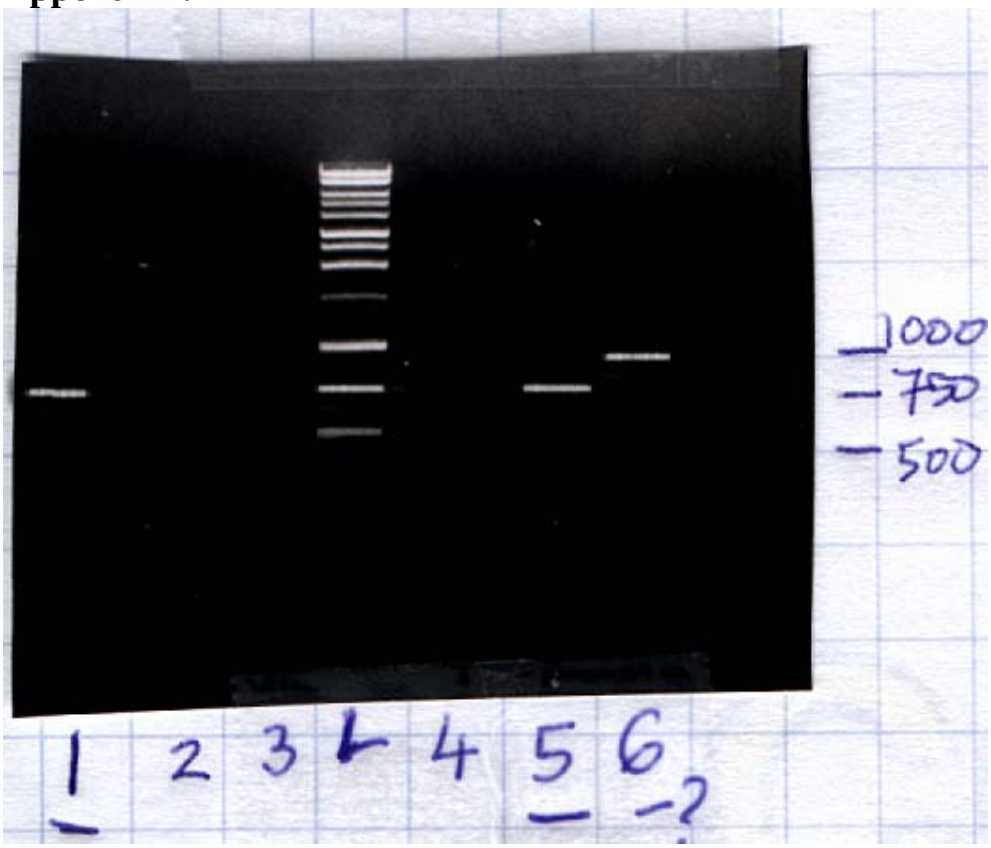
PCR results from transformed cells.
#10 shows positive insert size.

Appendix H.



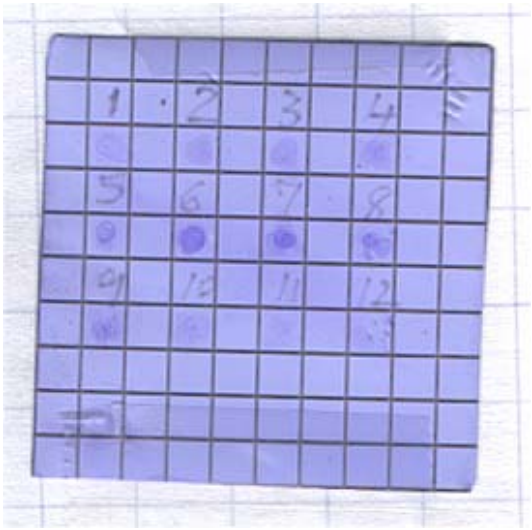
Enzyme digest on other transformed *E.coli* cells with plasmid.
Plasmid found empty.

Appendix I.



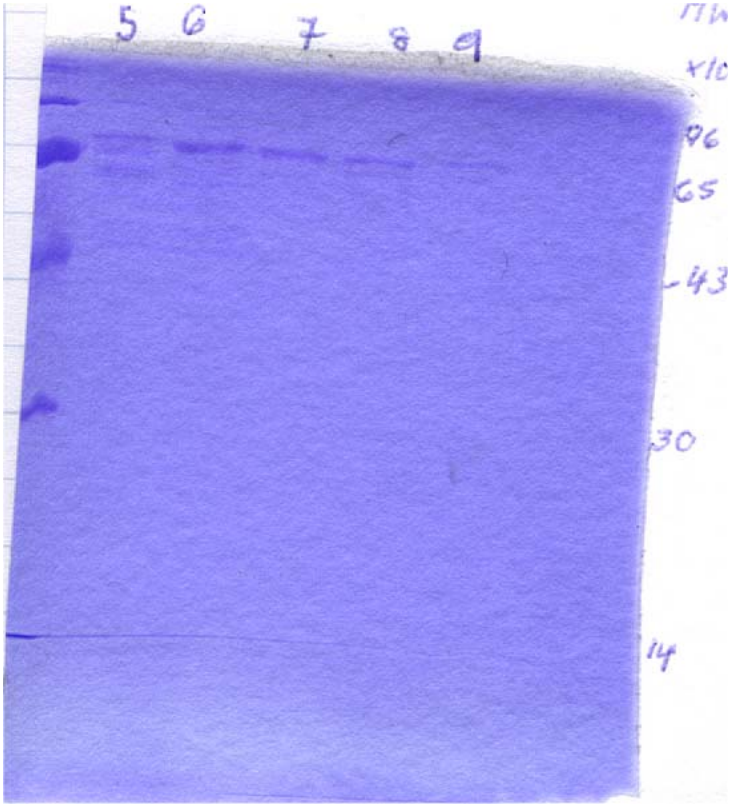
PCR product from smear and spot of colony #10 (positive)
#1 and #5 positive, with #6 insert slightly too big.

Appendix J.



Paper that tests for presence of a protein, with the presence marked by a dark blue spot that remains even after the dark blue dye is washed out.

Appendix K.



Example of Protein gel run on sample protein IF2 from IMAC