Insertion of the Escherichia Coli Phosphofructokinase A (PFKA) Gene into a plasmid, pRSETA

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Biology 600
Abstract:

We attempted to insert the Escherichia Coli (E. Coli) gene for Phosphofructokinase A (PFKA), an enzyme involved in glycolysis, into the plasmid pRSETA, place it under the control of the easily-inducible T7 RNA Polymerase, and his-tag the gene. We first designed appropriate primers and used PCR to amplify the PFKA gene with added restriction sites (HindIII and BamH1) at either end from several different DH5α K12 chromosomal DNA samples, some of which we had prepared ourselves. We then cut the gene, along with a sample of pRSETA, with the restriction enzymes HindIII and BamH1. Then we “gel purified” the resulting DNA, a mix of unwanted smaller fragments as well as the properly designed cut plasmid and gene. We then ligated the gene and the plasmid together, and chemically transformed the resulting ligation into DH5α competent cells. After screening numerous colonies for the presence of plasmid DNA using a miniprep and a restriction analysis using HindIII and BamH1, we had hoped to find evidence of at least one successful transformation of the pRSETA plasmid with the PFKA insert. However we have come up with no evidence of a successful clone, and after redoing the procedure many times as well as eliminating many sources of error, we have decided to abandon the project with the conclusion that the cloning of this gene is very difficult or impossible to carry out by our limited methods.

Introduction:

Over the summer, Kanyi, Dr. Hagler, and I decided we wanted to work together on a rational design project. Rational design involves creating specific changes in an enzyme’s structure in order to change its enzymatic properties in a directed way. We would then assay, or run specific kinetic (how fast does a reaction happen over time?) experiments on, the mutated enzyme. All we needed was a good enzyme to experiment on, two good organisms (one to get the gene out of, and one to express the protein in) to work with, and a good plasmid to “clone”, or insert the enzyme’s gene into.

Phosphofructokinase A, or PFKA, was an extremely good candidate for our experiment for many reasons. First, it is involved in an extremely common and ancient cellular process (glycolysis, or the splitting of glucose into two pyruvate molecules which “feed” the Krebs cycle). This would mean that we would have many potential organisms to work with, and the structure of PFKA would be fairly “conserved”, or very similar, among all organisms. Second, PFKA is a heavily allosterically regulated enzyme, meaning that there are many molecules that, if present in the reaction sample, affect the rate of reaction (the kinetics) of PFKA’s main reaction (the transfer of a phosphate ion from ATP to D-fructose 6-phosphate):

\[
\text{D-fructose 6-phosphate} + \text{ATP} \rightleftharpoons \text{D-fructose 1,6-biphosphate} + \text{ADP} + \text{H}^+ 
\]
These molecules, called inhibitors (slow down the reaction) and activators (speed up the reaction), bind to “allosteric sites” on the enzyme, changing the shape of the “active site” where the main reaction is going on, and thereby changing the reaction rate of the main reaction. E. Coli DH5α K12 PFKA is inhibited by Phosphoenolpyruvate (O₂C-CPO₃=CH₂), but activated by ADP and other diphosphonucleotides (Swiss-Prot, unknown date). With all its allosteric sites as candidates for designed changes in the enzyme, we would have more choice and flexibility in what to change in the rational design process. Lastly, the conversion of D-fructose 6-phosphate to D-fructose 1,6-biphosphate by PFKA is a highly exergonic (ΔG = -5.3 kcal/mol) (Bio 560 supplement, 2001) process that is usually carried out at just slightly above normal room temperature, meaning it would be easy to work with in the lab under fairly standard conditions.

After having chosen a protein to work with, we had to decide upon two organisms to work with, one which we would extract the PFKA gene from, and one which we would grow the protein in. We chose to extract the gene from E. Coli DH5α K12, because there was a readily available 3D structure of it’s PFKA on the NCBI Structure Database <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Structure> (#1883) (Shirakihara Y, Evans PR., 1988) with it’s associated inhibitors and reaction products shown attached to the protein. This structural data would be absolutely essential when redesigning the protein, as we would use it to see which amino acids to change in order to create a desired effect. For example, if we wanted to change how ADP inhibited PFKA, we would want to look at and change only the amino acids involved in this inhibition, namely the ones making up the allosteric site which bind to the ADP. Without our 3D model of the Protein, we would be lost to find out where the allosteric site is and which amino acids constitute the allosteric site. The next step would be to grow the protein in E. Coli BL21(DE3)pLysS. This organism was perfect for growing E. Coli DH5α K12 PFKA for three reasons. First, since the two strains of E. Coli are so similar, there would be few compatibility issues with the one’s gene being expressed in the other (i.e. the PFKA would most likely solubilize and fold properly under similar conditions inside the new strain’s cytosol). Second, E. Coli BL21(DE3)pLysS is optimized for producing large amounts of protein. Lastly, E. Coli BL21(DE3)pLysS is optimized for use with the T7 RNA Polymerase. RNA Polymerase makes an RNA copy of all the DNA “downstream” of it’s binding site, or “promoter” in a process called transcription. Then, ribosomes from the cell begin to turn these RNA instructions into protein at the start codon (ATG) in a process called translation. E. Coli BL21(DE3)pLysS is engineered to produce almost no T7 RNA Polymerase when not in the presence of isopropyl β-D-thiogalactoside (IPTG). So when IPTG is introduced into a culture of E. Coli BL21(DE3)pLysS, the T7 RNA Polymerase is produced in large quantities, and any gene that depends on T7 RNA Polymerase for it’s transcription is transcribed, and therefore, translated into protein in large amounts. So if we place the PFKA gene under control of the T7 promoter and insert it into E. Coli BL21(DE3)pLysS, we can control the expression of Phosphofructokinase A enzyme very easily by using IPTG (Invitrogen, unknown date).
In order to express the DH5α K12 PFKA gene as a protein, we needed to “clone”, or insert it, into a “vector”, which would provide us with the machinery, such as a RNA Polymerase promoter (binding site), to express the gene. We chose the plasmid pRSETA. Plasmids are circular pieces of non-chromosomal DNA that are found in many bacteria and plants. They are inheritable just like regular chromosomal DNA, and are fairly cheap and easy to work with in the lab. Plasmid pRSETA attracted us for many reasons. First, it had our T7 RNA Polymerase promoter (binding site), meaning it would work with our E. Coli BL21(DE3)pLysS induction scheme. Second, it had a his-tagging site. The his-tag site, as it’s name suggests, adds a string (in this case 6) of Histidine amino acids to the beginning or end of any protein inserted right before or after it. We would insert the PFKA gene into the pRSETA plasmid in such a way that the beginning of the gene was directly preceded by this his-tag site. This his-tag is useful because it provides a simple and cheap way to purify the his-tagged protein. The “his-tag” tends to bind with the metal ions Co++ and Ni++, and the procedure for purifying it takes advantage of this fact and is relatively simple, cheap, and easy to do. Third, pRSETA confers ampicillin resistance, meaning it makes bacteria which contain it immune to the effects of ampicillin, a common antibiotic which works by blocking the synthesis of the bacterial cell wall. This is useful when doing transformations (the insertion of the plasmid into the bacteria) with pRSETA, because one can easily select for bacteria which have received the plasmid by introducing ampicillin into the growth media. Lastly, pRSETA has a cloning site, which is useful when trying to insert a gene into it (cloning sites and transformations will be further explained later in the introduction) (Invitrogen, unknown date).

We tried to produce a pure form of the pRSETA plasmid with a E. Coli DH5α K12 PFKA gene “inserted” into it in order to use it as a template for large-scale production of active, purifiable E. Coli Phosphofructokinase A protein in E. Coli BL21(DE3)pLysS bacteria. Once we were able to make large amounts of pure PFKA easily by using E. Coli BL21(DE3)pLysS as an inducible “factory”, we could then do assays on PFKA to ascertain its enzymatic properties. As explained previously, we would then produce a series of rationally designed (using the 3D Model from Shirakihara Y and Evans PR.) mutant PFKA proteins using site-directed mutagenesis, and assay the mutant’s properties as well in order to test different theories of enzyme kinetics.

In order to produce large amounts of the pRSETA plasmid with a E. Coli DH5α K12 PFKA insert, we would have to produce large amounts of both pure plasmid and pure gene, and then insert the gene into the plasmid somehow. Pure plasmid is easy to obtain commercially, while obtaining a pure sample of PFKA that is able to be inserted into pRSETA requires the use of the Polymerase Chain Reaction (PCR). PCR makes billions of copies of a certain piece of DNA present in a sample very accurately and within a few hours. PCR is performed within a machine called a thermal cycler, which is able to heat and cool samples
of DNA very rapidly (PCR takes advantage of temperature changes in the sample). PCR occurs in three stages: DNA denaturation, primer annealing, and DNA extension. In DNA denaturation, the sample DNA is brought up to 95°C, denaturing the normally double-stranded DNA into two complimentary single strands. In the primer annealing, the sample of single stranded DNA is brought down to a temperature low enough (usually 50-60°C) so that “primers” or short oligonucleotides 20-40bp in length can bind to complimentary sequences in the sample DNA. These primers are usually designed long enough so that they only match one sequence of DNA in the sample. One can insure his or her primers are long enough by computing the probability of finding a complimentary sequence somewhere else in the sample DNA ((2*# of bp in sample)/4^(# of bp in primer)). As long as the primers are long enough, this probability should be low enough to ensure only the desired part of the sample is amplified. In most PCR setups, there are two primers, one for each strand of complimentary DNA. In the next and final step, DNA elongation, the sample is heated to 72°C, and a high-temperature DNA Polymerase “latches on” to the primers and starts copying the DNA sample in one direction only (5’ end to 3’ end), using up the free base pairs included in the PCR solution. Repeated many (usually 50-70 times) times over, these cycles produce a copy of the specific part of the DNA sample. As will become more clear in our example, the primers mark the “stop” and “start” points for copying the DNA sample. A good example of PCR can be found at the Virtual Bacterial ID Lab – PCR Amplification from Howard Hughes Medical Institute at <http://www.hhmi.org/biointeractive/vlabs/bacterial_id/shockframe/shockframe_a2.html>. Using PCR, we were able to quickly and easily create large amounts of the PFKA gene by using a pure E. Coli DH5α K12 chromosomal DNA sample, which we prepared ourselves.

The way we were going to insert our PFKA gene into pRSETA was by cutting both with two separate restriction enzymes, and then “ligating” the “sticky ends” back together. Restriction enzymes recognize certain patterns of DNA and cut them in a specific way (depending on which enzyme is used). They are originally derived from bacteria which had evolved them as a defense against invading viruses (cutting the virus DNA would make it impossible for the virus to insert itself into the bacteria’s genome and “hijack” the cell). For Example, the two enzymes we used, BamH1 and HindIII, recognize distinct patterns in DNA and cut it in a specific manner (denoted by |):

HindIII:  
| AGCTTXXXXX | XXXXG|GATCCXXXXX  
| XXXXTTCTGA|AXXXX | XXXXCCTAG|GXXXX

Where X can be any of the four base pairs. As you can see, the cutting creates a 4-base pair “overhang on either strand of DNA that we call a “sticky end””. When one ligates, or joins together, two pieces of restriction-enzyme-cut DNA, only strands with complimentary sticky ends will successfully ligate. To do a ligation,
we use an enzyme called, appropriately, ligase. We chose BamH1 and HindIII because they were included in the pRSETA multiple cloning site, essentially a string of DNA containing sequences recognized by many common restriction enzymes, and because HindIII and BamH1 recognition sites (as seen in the figures) are not to be found in the wild PFKA gene.

After our PCR is finished, we would like some way of checking to see that all went well with our PCR. We do this by performing gel electrophoresis on the sample. Gel electrophoresis is a technique whereby we are able to visualize the DNA contained in a sample of DNA, such as our finished PCR reaction, by sorting it by size. It uses a DNA-binding UV-fluorescent Dye called Ethidium Bromide, and an agarose gel, which acts like a molecular sieve to sort the fragments by size when a current is passed through the gel (which is made of cross-linked pieces of agarose polymer in electrolyte buffer solution), which is submerged in the same electrolyte buffer solution as in the gel to carry the current through and to the gel. The negative phosphate backbone of the DNA is attracted to the positive pole of the gel, and the DNA sorts by size due to the greater friction on the larger pieces, which makes them move slower than the smaller pieces. A 2-log DNA Ladder composed of fragments of DNA of a known size is added in a well adjacent to the sample wells so that the sizes of the unknown DNA fragments can be determined by referencing them to the ladder. Next, the gel is visualized by putting it under a UV lamp or transilluminator, and the glowing lines on the gel mark the presence of Ethidium Bromide, and therefore, DNA. After we have confirmed that our PCR sample contains only our desired PCR product (by looking for it and any impurities on the gel), we can continue on to ligating it with pRSETA.

After we PCR the PFKA gene and get our cutable PCR product, the “obvious” next step is to ligate the cut PCR product to the cut pRSETA plasmid. However, we first must remove the extra pieces of DNA (the two pieces of DNA that we cut off at the ends of the PCR product, and the stuff in between the BamH1 and HindIII restriction sites on the pRSETA plasmid) with BamH1 and HindIII sticky ends so that these pieces do not ligate to the pieces we want to ligate together (the cut pRSETA and the cut PCR product). We do this by a process called gel purification. Gel purification involves performing gel electrophoresis on a sample of DNA (in this case the pRSETA and the PCR product), and then cutting out the pieces of the gel containing the desired fragments (in the example, 5, 7, and 8 are all cut PFKA PCR products, and pRSETA is cut also):
Next, the DNA is extracted from the gel by spinning it in a purification column, which separates the agarose and the buffer/DNA by passing the gel through glass wool, which allows DNA and buffer to pass, but not agarose. The DNA is then precipitated with salt and ethanol, washed, and then purified. After Gel Purification is performed on the cut PCR product and cut pRSETA plasmid, we can then go ahead and ligate the two together with ligase.

Once we have ligated the wanted PCR product and pRSETA fragments together, we now need to transform the plasmid into bacteria in order to amplify (or produce greater quantities of) the plasmid, obtain a source of genetically identical PFKA/pRSETA plasmid, as well as further purify the ligation. Transformation is a process whereby we insert a piece of plasmid DNA into a bacteria. We use chemical transformation in our experiment, since it is both cheap and easy to use. It involves taking competent (i.e. transformable) E. Coli DH5α K12 bacteria, stored at -80°C and rapidly heating them and cooling them while in the presence of the desired DNA and Ca++ ions. While nobody knows for sure how this process works to transform the bacteria, one theory says that the rapid heating and cooling produces small holes in the cell membrane of the bacteria, opening up spaces for the plasmid DNA to pass through. After transformation, the bacteria are plated onto LBamp media, on which only those bacteria which have taken up the plasmid will survive (since pRSETA confers ampicillin resistance), selecting out bacteria which have not been transformed. After allowing the plates to grow overnight, single genetically identical colonies (the progeny of one single transformed cell) are picked from the plate and grown in LBamp overnight as to continue the ampicillin selection pressure. The tubes now contain genetically identical bacteria with genetically identical PFKA/pRSETA plasmids. Next, DNA is extracted from the bacteria in the tubes by means of a miniprep, which bursts the cells with lysozyme (which pokes holes in the cell membrane), and by boiling...
the bacteria, destroys many contaminants in the sample, such as RNA, and isolates the plasmid DNA. Next, the PFKA/pRSETA sample is “restriction analyzed”, as to insure that the PFKA PCR product has successfully been inserted into the pRSETA vector. In this case, restriction analysis involves cutting some of the miniprep plasmid DNA with BamH1 and HindIII, and then running a gel on the resulting digested miniprep. By using our knowledge of the plasmid we created (i.e. that it has only one HindIII site and one BamH1 site), we can determine the expected fragment sizes for the PFKA/pRSETA plasmid after they have been cut, and look for those fragments on the gel to determine which minipreps contain large amounts of pure, genetically identical PFKA/pRSETA plasmid. By performing this additional step of transforming E. Coli DH5α K12 with our ligation, we ensure that the DNA we use to transform the E. Coli BL21(DE3)pLysS (our “protein factory”) with is a large sample of pure, genetically identical plasmid.

However, as we continued to repeat the cloning procedure, and continued to see it fail, we began to try to troubleshoot the procedure. One thing we did was to restriction analyze the DNA inside the tube we thought contained pure pRSETA, as well as a miniprep from one of our colonies that did not contain the insert, and a sample of the gel purified cut pRSETA we were using. We ran a restriction analysis with three different enzymes – TspR1, Tsp5091, and TaqI. Based on how we designed our insert and cloning procedure, we came up with maps of where we knew the specific enzyme was going to cut on the three samples, and then looked for the theoretical fragments on our gel when we ran the digests (see appendix D). The absence or presence of these fragments would tell us whether or not the samples actually contained pure pRSETA or not.

Another thing we did was to decrease our pRSETA vector concentration in the ligation by only gel purifying 1uL instead of 5ul. This way, we would possibly increase the efficiency of our transformations by creating a higher percentage of clones with insert (if too much pRSETA DNA was present compared to the amount of cut PFKA vector, many amp-resistant plasmids could be created by just be religating the sticky ends of the cut pRSETA vector by some unknown process without inserting the PFKA vector, or the cut pRSETA vector could dimerize and religate in pairs, theoretically reducing the percentage of screened clones with insert.)

We also purified one of the PCR samples (PCRB2) before we cut it using QIAGEN’s QIAquick Spin protocol to see if that would help our experiment by removing any impurities (possible sources of error). The QIA quickspin kit relies on the fact that only large (100bp-10,000bp) DNA fragments bind to certain types of silicates (provided in the kit) in certain solvents (also provided). When the PCR product is dissolved in the correct solvents and passed through the silicate membrane, the impurities (primers, in our case in the 30bp range, and Chromosomal DNA, in the 4Mbp range, as well as other various chemical impurities including the rTAQ and nucleotides used in the PCR mix) pass right
through and only the desired fragments (in our case, the ~1000 bp PCR fragment) stick to the membrane. The membrane is then washed with a solvent that “unsticks” the desired fragments from the membrane, thereby purifying the PCR product (QIAGEN, unknown date).

In the winter we also checked to see if the HindIII and BamH1 sites were cutting by running diagnostic XbaI/HindIII and XbaI/BamH1 digests. XbaI lies to the left of the Multiple Cloning site, at the ends of which the BamH1 and HindIII sites are located. Again, we created maps of what we should expect if the sites were there (see appendix D) and in fact, they were.

As a last resort when we did not see that our second set of colonies for the winter term was not showing us insert, we decided to run more diagnostic restriction analyses (PvuII/XbaI and NdeI) on the minipreps we created. Again, from the maps we created of pure pRSETA plasmid, our theoretical clone, a third plasmid created by religating by unknown method the noncomplimentary sticky ends of the cut and purified pRSETA vector, and the suspected pRSETA dimer, we predicted the fragments we would see for each case (see appendix D). However, the results were nonconclusive.

Methods:

1. **DNA EXTRACTION**

First, obtain a sample of E. Coli DH5α K12 bacteria, and incubate in 3 mL LB broth (in 15mL Eppendorf tubes – keep caps at half-open position in order to aerate sample) overnight at 37°C in an orbital shaker. Next, follow this DNA extraction procedure:

1) Aliquot 0.5mL of bacterial culture from the LB into a 1.5mL Eppendorf tube. Spin in a micro-centrifuge at maximum speed for 5 minutes. Remove and discard supernatant.
2) Add the following solution to each tube:
   i) 451.2uL of 10mM Tris-HCl and 441.2 uL of distilled water
   ii) 20uL of .25M EDTA
   iii) 23uL of 10% SDS
   iv) 3.8uL of Proteinase K (20mg/ml)
3) Incubate tubes at 37°C for 1 hour
4) Extract DNA with phenol-chloroform-isooamyl alcohol solution twice: Vortex the tubes and add 0.5mL of the phenol-chloroform-isooamyl alcohol solution. Vortex the tubes again, and spin them for 1 minute at maximum speed in a micro-centrifuge. Remove the upper layer and save it in a separate tube. Add another 0.5mL of the phenol-chloroform-isooamyl alcohol solution to the bottom layer, and spin again for one minute at maximum speed in a micro-centrifuge (This will separate out even more DNA). Again, retain the upper
layer by adding it to the previously saved upper layer (from the earlier step). The upper layer contains the DNA. Now discard the lower organic layer.

5) Extract the DNA with chloroform solution. Add 0.5mL of chloroform to each 1.5mL Eppendorf tube, vortex each tube, and then spin them for one minute at maximum speed in the micro-centrifuge. Again, discard the lower organic layer and retain the upper aqueous layer containing the DNA.

6) Add 50uL of 5M NaCl solution to the upper layer that has been retained, and then vortex each tube.

7) Add 0.3mL of isopropanol to each tube and gently invert.

8) Spin 10 minutes at maximum speed in a micro-centrifuge. Carefully remove supernatant, avoiding nucleic acid pellet at bottom of tubes.

9) Wash pellets with 0.5mL of 70% ethanol. Invert gently and spin for 5 minutes at maximum speed in micro-centrifuge. Carefully remove ethanol, avoiding nucleic acid pellet at bottom of tubes.

10) Allow pellets to air dry overnight by opening the tubes and letting them sit at room temperature.

11) Re-suspend pellet in 50uL of the following mixture:
    i) 10 uL of 1M Tris-HCl
    ii) 2uL of .5M EDTA
    iii) 1mL of sterile water

12) Allow nucleic acids to solubilize overnight

2. PCR

Notes on Designing Primers:

Our strategy for inserting PFKA into pRSETA would be to design the primers so that the PCR product contained a BamH1 and HindIII site on either end, so we could cut the gene and make the proper “sticky ends”, complimentary to our pRSETA “sticky ends”, without cutting the gene into pieces (because the actual gene doesn't have any HindIII or BamH1 sites on it). Also, the two enzymes work under similar conditions, enabling us to do a simultaneous “double digest” on our PFKA PCR Product and pRSETA.

With our strategy in mind, we had to design primers so that several conditions were met. First, the PCR product must place the PFKA gene, when cut by restriction enzymes and ligated, in the same reading frame as the plasmid DNA. Second, the PCR product must have the two restriction sites (HindIII and BamH1) at either end of the PFKA gene. Third, since restriction enzymes are most effective when there is at least some DNA on either side of the recognition site (i.e. some XXXX, as in the diagram), the PCR product would have to have its restriction sites slightly “inlaid” from the end of the strand. Fourth, the “sense” strand of the PFKA gene would have to be on the same side, or strand, as the “sense” strand of the plasmid. Fifth, and most obviously, the primers must selectively copy and bind to the PFKA gene. To make sure all of these conditions were met, we needed both the sequence of the pRSETA plasmid and
the PFKA gene in E. Coli DH5α K12. While the pRSETA sequence was easy to obtain (it was included in the shipment of the plasmid, and can be found online at <http://www.invitrogen.com/content.cfm?pageid=7471>), we had to search online for the sequence of the PFKA gene in E. Coli DH5α K12. We searched the NCBI Nucleotide Database <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide> for this gene and promptly found it (type in “2367328” in the “search” box). Now let’s look at the “left” side, or 5′ end (sense strand) of our gene and design a primer so that all 5 goals are met – we will look at the pRSETA sticky end we are trying to match as well as the antisense strand of the PFKA gene (because RNA Polymerase elongates 5′ to 3′ only, we need the 5′ primer to bind to the antisense strand):

**SPACES DENOTE CODONS, | denotes a cut**

<table>
<thead>
<tr>
<th>pRSETA (BamH1 cut)</th>
<th>PFKA (antisense single strand)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense:</td>
<td>Antisense:</td>
</tr>
<tr>
<td>5′ TGG G</td>
<td></td>
</tr>
</tbody>
</table>

Let’s let our left hand primer bind to 21 nucleotides of the PFKA gene. Using our formula for probability … \((2\times4.6\times10^6)/4^{21}\)=2.1x10^-6, we see that it is two in a million that we would inadvertently copy another sequence in the E. Coli genome as well. Given the reference frames of the two pieces of DNA, and the fact that we need a BamH1 “sticky end” cut, and some room on either side of that cut on the PCR product, we get the following design for a primer (while the primer “sticks out” in the first cycle of PCR, later copies are the same length as it):

**pRSETA (BamH1 cut) / 5′ Primer (sense)**

<table>
<thead>
<tr>
<th>S:</th>
<th>A:</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′ TGG G</td>
<td>3′ ACC CCT AG</td>
</tr>
<tr>
<td>X XXX GA TCC ATG ATT AAG AAA ATC GGT GTG 3′</td>
<td>TAC TAA TTC TTT TAG CCA CAC…5′</td>
</tr>
</tbody>
</table>

In our case the XXXX was TTCC. Notice how the PCR product’s 5′ end (sense) will look now when it is cut, and how it will satisfy all the 5 requirements:

**pRSETA (BamH1 cut) / PCR Product**

<table>
<thead>
<tr>
<th>S:</th>
<th>T:</th>
<th>A:</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′ TGG G</td>
<td>T TCC GA TCC ATG ATT AAG AAA ATC GGT GTG…3′</td>
<td>A AGG CCT AG</td>
</tr>
<tr>
<td>X XXX G</td>
<td>G TAC TAA TTC TTT TAG CCA CAC…5′</td>
<td></td>
</tr>
</tbody>
</table>

Primer Design for the 3′ end is very similar (sequence shown is 13bp downstream of stop codon TGA, see Appendix A):

**PCR Product (primer is antisense and 33bp long, original gene sequence highlighted)**
S: 5’…CA GAT TCC TTT ACC CTG AAA CA|AGC TAT GCA T 3’
A: 3’…GT CTA AGG AAA TGG GAC TTT GTT CGA |ATA CGT A 5’

pRSETA (cut) 3’ end (sense)

S: 5’ |AGC TTG ATC…3’
A: 3’ |AC TAG…5’

Notice that it doesn’t matter if the frame stays the same, the stop codon has already terminated transcription, and the reference frame doesn’t matter to the T7 RNA Polymerase, it detaches along a certain sequence regardless of the reading frame. Sequences of the pRSETA plasmid (multiple cloning site and “tag” only) and PFKA gene are contained in Appendix A.

Lab Procedure for PCR:

Dilute the following primers to 50mM concentration:

PFKA 3’ Primer: 5’ ATGCATAAGCTTTCTAGGTAAGGTAATCTG 3’
PFKA 5’ Primer: 5’ TTCCGATCCATGATTAAGAAAAATCGGTGTG 3’

Perform the following PCR procedure:

1) Using a fresh tip for each component, add 1uL of both 50mM primers to a 0.5mL PCR tube, along with 18uL distilled water.
2) Using a fresh tip, add 25uL of rTAQ premix to the water/primer mix
3) Now add 5uL of DNA sample from DNA extraction procedure to the 0.5mL PCR tube with a fresh tip.
4) Vortex the tube and pool reactants at bottom of tube.
5) Program the thermal cycler to perform the following profile:
   i) 5 minutes at 94°C
   ii) 60 cycles of 1 minute at 95°C, 1 min at 40°C, 2 min at 72°C
   iii) 10 minutes at 72°C
6) Freeze the tubes at -5°C until needed next

To check that the PCR was done ok, next run a 1% TBE agarose gel (wear gloves when handling gels because Ethidium Bromide is a suspected carcinogen):

1) Aliquot 5uL of the 40uL PCR sample into a 1.5mL Eppendorf Tube, and add 1uL of 6x Loading dye. Put on ice until ready to load the gel.
2) Weigh out 0.4g agarose
3) Place in a 125mL Erlenmeyer flask
4) Add 40mL 1XTBE (Tris-Borate-EDTA)
5) Microwave for about 45 seconds, or until sample begins to boil
6) Mix and repeat step 5 until solution is clear, and agarose is completely dissolved
7) Let the sample cool until you can comfortably place a finger on the side of the flask for more than 30 seconds
8) Add 2.5ul 10mg/ml Ethidium Bromide and swirl gently to mix
9) Pour the gel into the gel tray and put the combs in place
10) Allow the gel to cool and harden, it will become pale blue and solid when ready
11) Remove the combs and reorient the gel tray in the gel box so the combs are parallel to the top of the box.
12) Submerge the gel in 1xTBE
13) Load 1ul 2-log ladder into one well
14) Load 6uL PCR sample to another well
15) Run the gel with the negative anode on the same end as the wells and the positive cathode at the other end.
16) Run the gel at 150-180V and .25Amps for about 20 minutes, or until the purple loading dye is 2/3 of the way down the gel.
17) Remove the gel from the box and place it on the UV transilluminator
18) Turn the box on and look at the gel with the cover closed
19) Take a picture of the gel with the Polaroid camera by lifting the gel cover and then placing the camera in the right orientation on top of the gel box. Depending on whether the gel looked really bright or faint, take a 1-3 second exposure, and allow it to develop for 15 seconds before you open it.
20) Throw the gel in the trash

Look at the picture of the gel and look for the ~1000 bp PFKA fragment. If it is not visible, try the PCR again under different conditions. If it is visible, continue on to Restriction and Gel Purification.

Lanes 8, 5, and 7 have the appropriate ~1000bp PFKA fragment:
3. Restriction and Gel Purification

Next, set up a double HindIII/BamH1 double digest on the PCR sample and a sample of commercially available pRSETA plasmid (Invitrogen) according to the following protocol:

1) Add the following solutions in order as listed to each tube, making sure to use a fresh tip for each ingredient:

<table>
<thead>
<tr>
<th>TUBE 1</th>
<th>TUBE 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5uL Enzymatic-grade water</td>
<td>4uL Enzymatic-grade water</td>
</tr>
<tr>
<td>2uL 10xNE Buffer 2</td>
<td>4uL 10xNE Buffer 2</td>
</tr>
<tr>
<td>.5uL HindIII</td>
<td>1uL HindIII</td>
</tr>
<tr>
<td>.5uL BamH1</td>
<td>1uL BamH1</td>
</tr>
<tr>
<td>5uL pRSETA plasmid</td>
<td>30uL PCR sample</td>
</tr>
</tbody>
</table>

2) Close tubes, vortex, and then pulse in minicentrifuge to pool ingredients at bottom of tube.
3) Place in 37°C water bath for 40 minutes
4) Freeze tubes for later use at –5°C or continue on to gel purification

Now follow the gel purification protocol:

1) Set up a 5-well 40mL 1% agarose gel as in the PCR checking procedure and load 1uL 2-log ladder into one well, 20uL digested pRSETA sample with 4uL 6xLoading Dye in another well, and 40uL digested PCR sample with 8uL 6xLoading Dye in another well.
2) Run the gel at 150-180V at .25Amps for 20 minutes or until loading dye is 2/3 of the way down the gel.
3) Look at the gel under the UV Transilluminator and take a picture.

Gel should look something like this (lanes 5, 7, and 8 are all identical digested PCR samples):

![Gel Image]

4) Cut out the part of the gel containing the ~5000bp pRSETA band with a razor blade and put it into a labeled purification column. Cut out the part of the gel containing the digested PCR sample’s ~1000bp band with a razor blade and put it as well in a labeled purification column (see end of procedure for this section for procedure on making purification columns).
5) Spin the purification column in a microcentrifuge at full speed for 45 seconds, and discard the smaller tube nestled inside the bigger tube with glass wool and agarose in it, being careful not to spill the sample of liquid containing the DNA in the larger tube:

![Diagram of Centrifuge]

6) Estimate the volume of the liquid in each tube and then add 1/10 volume of 5M NaCl, and 2½ volume 100% Ethanol to precipitate DNA.
7) Spin for 10 minutes at full speed in a microcentrifuge
8) Carefully remove all but bottom ~10ul of sample, trying not to disturb that bottom layer
9) Resuspend ~10uL sample in 300uL 100% Ethanol and vortex.
10) Spin sample for 2 minutes at full speed in a microcentrifuge
11) Carefully remove all but bottom ~10ul of sample, trying not to disturb that bottom layer
12) Evaporate overnight at room temperature or use a vacuum chamber or dessicator to dry quickly at room temperature.
13) Resuspend the dried DNA pellet in 10uL Enzymatic-grade water and vortex for 1 minute or more to make sure DNA is fully solubilized.

Procedure for making one purification column:

1) Cut the tops off of one 1.5mL Eppendorf tube and one 0.5mL PCR tube.
2) Poke a small hole (~1mm in diameter) in the bottom of the PCR tube using a sharp, sterilized object such as a safety pin or dissecting probe (to sterilize and make the poking easier you might want to heat the instrument red hot before making the hole in the PCR tube – make sure to do this in a well ventilated area as plastic may smoke a little)
3) Place a small wad of balled-up glass wool at the bottom of the PCR tube and push down so that it seals the hole and takes up approximately the bottom ¼ of the tube
4) Place the PCR tube with the hole and glass wool in it inside the 1.5mL Eppendorf tube. The column should look like this:

```
  Glass wool
  0.5mL PCR Tube
  1.5mL Eppendorf Tube
```

4. **Ligation**

Now that we have pure samples of the cut plasmid and cut PFKA gene fragments that we want, we can go ahead and ligate those fragments together to make the complete plasmid:

1) Add the following components in order to a 1.5mL Eppendorf tube:
   i) 2.5uL Enzymatic-grade water
   ii) 1ul 10xLigase Buffer (when thawing the buffer, a white precipitate may develop, vortex the tube until the precipitate is completely dissolved)
   iii) 1ul cut pRSETA and 5uL cut PFKA DNA
   iv) .5uL Ligase
2) Pulse in a microcentrifuge to pool the reactants, and let the mixture sit overnight at room temperature

5. **Transformation of the Ligation into E. Coli DH5α competent cells**

Now that we have a ligation, we need to further purify it by transforming bacteria with the ligation, and selecting for the presence of the pRSETA plasmid with ampicillin:

1) Aliquot 50uL COLD (i.e. just melted from the –80°C freezer) competent DH5α cells into a 1.5mL Eppendorf tube and immediately place on ice.
2) Add 3uL of the ligation to the tube and pulse in a minicentrifuge if needed to pool the cells and the ligation together.
3) Let the ligation/competent cells sit on ice for 5 minutes
4) Heat shock the cells in a 37°C water bath for 20 seconds
5) Immediately put on ice for at least 2 minutes
6) Add 900uL room temperature LB Broth to the tube, and incubate the tube at 37°C for 15 minutes
7) Spin the cells for one minute at full speed in a microcentrifuge to get the cells to the bottom of the tube (cell pellet).
8) Remove 550mL of the supernatant, and vortex the tube to resuspend the cell pellet
9) Plate the remaining 400mL on two LBamp plates, 200mL each, using a sterile spreader.
10) Incubate the plates overnight at 37°C

6. **Picking, growing, and screening of Colonies**

Next, we will pick the colonies that grew on the plates (which have all been successfully transformed by the pRSETA plasmid, because they were able to grow on the LBamp plates) and test them for the presence of the PFKA insert in the pRSETA plasmid.

1) Aliquot 3mL LBamp into each of 40 15mL Eppendorf Tubes, labeled 1-40.
2) Pick 20 colonies (the big ones, not the smaller satellites) from each plate using a sterile toothpick or flame-sterilized nichrome wire and swirl around in one of the 40 15mL Eppendorf Tubes filled with LBamp.
3) Place the tubes in an incubated (37°C) orbital shaker running at 300rpm overnight. Make sure the caps are at the half-open stop in order to aerate the tubes.
4) Once the tubes are grown, check each tube to see if it is cloudy, indicating that bacteria grew in the tube. Put aside the tubes that didn’t grow, and follow the miniprep procedure for those that did.

Miniprep:
1) Pour 1.5mL of the 3mL LBamp contained in the 15mL Eppendorf tube into a similarly labeled (1-40) 1.5mL Eppendorf tube.
2) Spin the tube for 30 seconds at full speed in a microcentrifuge
3) Remove all the supernatant by just pouring it out, being careful to leave the cell pellet behind.
4) Resuspend the cells in 50uL Lysis buffer (10mM Tris-HCl pH 8.0, 1mM EDTA, 15% wt/vol sucrose, 2 mg/ml lysozyme, 0.2mg/ml pancreatic RNase, 0.1 mg/ml bovine serum albumin) this buffer can be stored at -20°C.
5) Boil the suspension for one minute and quickly put on ice for one minute
6) Centrifuge the tubes for 15 minutes at maximum speed in a microcentrifuge at room temperature.

Now we will cut the plasmid with restriction enzymes (BamH1 and HindIII), which, if the colony has the plasmid with the PFKA insert, will produce two fragments, one of ~1000bp and one of ~5000bp, which can be seen on a gel and then the miniprep can be used as a source of pure PFKA/pRSETA plasmid. If the colony does not have the insert in the plasmid, only the ~5000 fragment will show up.

Restriction procedure is as follows:

1) Add the following components, in order, to an appropriately labeled (1-40) 1.5mL Eppendorf tube, making sure to use a new tip each time:
   i) 12.5uL Enzymatic-grade water
   ii) 2uL 10xNE Buffer 2
   iii) .5uL HindIII and .5uL BamH1
   iv) 5uL of the supernatant from the appropriate miniprep (store the minipreps at -5°C when not being used)
2) Pulse the tube in a minicentrifuge if necessary to pool the reactants
3) Incubate the tubes at 37°C for 30 minutes

Next, we will run a gel on the restriction products to determine which colonies have the PFKA insert:

1) Add 4ul 6xLoading dye to the 20uL digests prepared earlier
2) Prepare a 40mL 1% agarose gel as in the PCR procedure
3) Add 1ul 2-log ladder to one well in each row of wells
4) Load the samples, record where each sample went on the gel, and run the gel at 150-180V and .25Amps for 20 minutes or until the purple loading dye is 2/3 of the way down the gel (if you are running two rows of wells, wait until the loading dye is almost to the next row of wells).
5) Look at the gel in the UV transilluminator, and take a picture of the gel using the UV Polaroid camera
6) Save the minipreps and 15mL Eppendorf tubes of bacteria that have the insert (~1000bp), and discard those that don’t.

7. General Formulas and Conditions for Restriction analysis of DNA
1) For a 20 uL digest, add the following to a 1.5 mL Eppendorf tube in order, making sure to use a fresh tip for each ingredient:
   i) 2 uL 10x Buffer
   ii) Enough ultrapure water that the sample’s total volume is 20 uL
   iii) .2 uL of 100x BSA if needed
   iv) .5 uL of each restriction enzyme
   v) DNA Sample

2) Pulse in a microcentrifuge to pool the ingredients at the bottom of the tube
3) Incubate for however long specified (usually no more than 30 minutes or 1 hour for minipreps and no more than 8 hours for pure DNA)
4) The buffer, enzymes you use should depend on the type of digest being done. Restriction digests and their appropriate conditions performed in this project were:

<table>
<thead>
<tr>
<th>Buffer</th>
<th>BSA?</th>
<th>Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HindIII/BamHI –</td>
<td>NE2</td>
<td>OPTIONAL 37</td>
</tr>
<tr>
<td>XbaI/PvuII –</td>
<td>NE2</td>
<td>YES 37</td>
</tr>
<tr>
<td>XbaI/HindIII –</td>
<td>NE2</td>
<td>YES 37</td>
</tr>
<tr>
<td>XbaI/BamHI –</td>
<td>NE2</td>
<td>YES 37</td>
</tr>
<tr>
<td>TspR1 –</td>
<td>NE4</td>
<td>YES 65</td>
</tr>
<tr>
<td>Tsp5091 –</td>
<td>NE1</td>
<td>NO 65</td>
</tr>
<tr>
<td>Taq I –</td>
<td>Taq I Buffer</td>
<td>YES 65</td>
</tr>
<tr>
<td>Nde I –</td>
<td>NE4</td>
<td>NO 37</td>
</tr>
</tbody>
</table>

8. QIAquick Spin Protocol for Purifying PCR products from 100-10,000bp

SEE APPENDIX C

or


Results:

Our First attempt at PCR Failed (Figure 1).

Figure 1: PCR Failure (lanes 1, 2, and 3 are all PCRs run from purifications of chromosomal DNA from different sources of E. Coli DH5α K12)
We then ran the PCR under identical conditions and were successful (Figure 2)

Figure 2: Lanes 1,2,3 and 4 are unsuccessful controls for the presence of chromosomal DNA using primers (16S ribosomal RNA primers, see Appendix B); 5,6,7, and 8 are all PCRs done with the PFKA primers and different sources of chromosomal DNA from DH5α K12 bacteria – 6,7, and 8 correspond to 1,2, and 3 in the failed PCR, lane 5 is a PCR done some chromosomal DNA previously prepared by another student

We then took the successful PCRs (5,7, and 8), and cut them along with pRSETA (see procedure). We then gel purified the samples (Figure 3)
Unfortunately, sample 7 blew up in the centrifuge (purification column) when we spun it, and so we were only able to ligate sample 5 with pRSETA. Due to time constraints, we only ligated for 90 minutes before we did our first transformation, and let the rest of the ligation go overnight. We screened the resulting colonies (Figure 4)

As evident from the photograph, only 10 colonies grew, and none of them contained the insert, so we reused the 30 LBamp broth tubes with nothing
growing in them and reseeded them with colonies from the plate (transformed after 90 minutes of ligation) (Figure 5):

Figure 5: Two photographs showing the same gel at different times (we ran it longer due to the interference of the loading dye band at ~1000bp when we first took it off the box) of minipreps digested with BamHI/HindIII (screened colonies)

Only 9 samples grew, and there was no evidence of an insert in any of them. So we threw out all the remaining LBamp broth tubes, and transformed, plated, picked, grew, miniprepped, cut, and ran the 24-hour ligation (Figure 6)

Figure 6: Cut (BamHI/HindIII) minipreps of the 24-hour ligation
In sheer awe and frustration on how much our minipreps failed in Figure 6, as evident by BARE TRACES, IF THAT, OF DNA on the gel, we dutifully retried our minipreps from the remaining LBamp we had kept in the refrigerator containing the picked colonies grown in the 15mL Eppendorf tubes (Figure 7)

Figure 7: Retries of the minipreps in Figure 6 (digested with BamHI/HindIII)

![Image of gel electrophoresis](image)

Obviously, even though our minipreps worked in Figure 7, our insertion was not to be found. Since we had failed in doing steps beyond the PCR, we started back at our PCR samples, and followed the procedure from there. After cutting the PCR product and pRSETA again, we ran another gel purification (Figure 8)

Figure 8: Second gel purification of pRSETA and PCRs
We ligated the PCR samples to the vector and transformed them, however, the plates dried out over winter break and we decided to start over from fresh PCRs anyways, given that they might have been the source of our problems fall term. We ran the PCR reaction on our 4 DNA samples again, and then ran them on a gel to see what, if anything, they contained (Picture lost). Only PCR sample 2 was successful. Next, we cut a fresh batch of pRSETA vector along with PCR2 with BamHI and HindIII and then gel purified the product (Figure 10). We cut the samples for 8 hours, instead of our usual 2-3 hours at standard conditions in order to insure that both samples were fully digested.

Figure 10: Gel Purification of new pRSETA and PCR2
As you can see in Figure 10, there was a lot of vector DNA compared to PCR product in the resulting gel purification product. Next, we ligated the two samples overnight, and then ran the ligation on a gel (Figure 11)

Figure 11: Ligation (LIG2) of pRSETA and PCR2 gel purifications in Figure 10

While the ligation in Figure 11 had an unexpected band at >10kbp, it was not of immediate concern. The faint band at around 3.9kbp is the band we were after as our assembled vector. Next, we transformed competent cells with the ligation, plated them, grew the colonies, miniprepped, and digested them with HindIII and BamHI to screen them for insert. We ran the digested minipreps on a gel (Figure 12)

Figure 12: Digested colonies 1-10
As evident in Figure 12, none of the colonies contained the desired insert. Frustrated, we decided to rule out the possibility that our supply of vector was contaminated. We ran a restriction analysis of plasmid samples contained in the gel purified and cut (BamHI/HindIII) pRSETA from Figure 10, as well as the supply of pure vector we were using for that digest, and one miniprep (Colony 6 in Figure 12). We digested with three enzymes, TspR1, Tsp5091, and TaqI. We hypothesized the fragments based on maps we had of the pRSETA plasmid and what the gel purified cut pRSETA product should look like. The results (in Figure 13) confirmed that all 3 samples were in fact pRSETA or our expected gel purified product (see appendix D for a “theoretical” digest).

Figure 13: Restriction analysis using 1=TspR1 2=Tsp5091 and 3=TaqI with a=Gel purified cut pRSETA product b=Pure pRSETA DNA supply c=Miniprep 6 from Figure 12
At a loss what to do, we decided to attempt a clone from scratch one more time, this time lowering the pRSETA vector concentration in the ligation mix by cutting and gel purifying less pRSETA DNA. We remade a sample of purified vector, this time only purifying 1µl of DNA instead of 5µl, and checked for the presence of both Hind III and BamH1 sites by doing a double digest with Xbal (Expected fragment sizes INSERT FRAGMENT SIZES) (Figure 14)

Figure 14: Testing for the presence of the HindIII and BamH1 sites with Xbal, and gel purifying pRSETA cut with BamH1 and HindIII

2-log a1 a2 a3 b1 b2 b3 c1 c2 c3
We then tested the gel purification product (B/H in Figure 14) to see if it contained the expected 2.9kbp fragment (Figure 15)

Figure 15: Testing the gel purification product for quality (B/H in Figure 14)

The fragment ran a little heavy in Figure 15, but it was not of concern, as the gel contours in Figure 15 were all wacky anyways. We then redid PCR on our E. Coli DH5α K12 chromosomal DNA samples (Figure 16)

Figure 16: Another PCR from our 4 sources of chromosomal DNA

2-log pRSET A
We then gel purified the samples, after QIAquick Spin purifying sample B2 to remove impurities to see if that would help, (Figure 17), and ran the gel purification products on a gel to test for quality (Figure 18)

Figure 17: Gel Purification of PCRs in Figure 16

Figure 18: Testing the products of gel purification (Figure 17) for quality, as well as the sample of pRSETA, which ran funny in Figure 15 (B2pur is labeled B2 to save space on the more narrow gel lanes)

While the 3 bands of the pRSETA vector would imply an incomplete cut or the presence of supercoiled, nicked, circular, and linear DNA types, we continued on to ligation after one more running of PCRB2pur, pRSETA, and PCR2 (just for fun to see what happened to it) (Figure 19)
Figure 19: Further testing of DNA samples

Note the <100bp band in the B2pur lane (hmmm... isn't that supposed to be pure???) and the triple band in the pRSETA lane (not good as well). Well, as it was our last attempt, and we had run out of ideas, we decided to ligate anyways, then transform, plate, pick, grow, miniprep, and screen. First, we screened all 40 colonies (1-14 were the result of ligating PCRB2pur and pRSETA, 15-27 were PCR3 and pRSETA, and 28-40 were PCRK and pRSETA) with our usual BamH1/HindIII digest scheme (Figures 20-22).

Figure 20: Colonies 1-9 screened twice on separate occasions with HindII/BamH1 double digest
As you can see, the only colony that has anything interesting going on in it is colony #34 (see Figure 22), however, the 2 fragments are of ~400bp in length and are therefore uninteresting for our cloning efforts. As you will also notice,
some of the minipreps had two bands (besides the chromosomal DNA band at the top of every lane -- see #10 etc… in Figures 20 and 21 with two bands) indicating some type of other product (see Discussion). Next, we performed two different sets of other restriction digests to screen for a clone in these minipreps, namely a XbaI/PvuII (Figure 23) digest and an NdeI digest (Figure 24).

Figure 23: XbaI/PvuII digest of minipreps 1-9

![XbaI/PvuII digest of minipreps 1-9](image)

Figure 24: NdeI digest of minipreps 25-40 (minus 32)

![NdeI digest of minipreps 25-40 (minus 32)](image)
The results from this additional analysis was not helpful at all in finding any colonies with insert, clarifying what the additional fragments were in miniprep #34 Figure 22, or in identifying exactly how the minipreps contained plasmids religated by unknown mechanism.

Discussion:

Our results tell us that we have successfully created the desired PCR product (as evidenced by a ~1000bp band on Figures 2, 9, and 16). We can confirm that both sites (HindIII and BamH1) on the plasmid from our pure supply of pRSETA were working (Figure 14), however, we have no confirmed ligation of the cut pRSETA and the PCR product. Lastly, while some of our minipreps may have contained the inserted plasmid (as would be evidenced on the restriction analysis gels by a band at ~3000 for the pRSETA fragment and a band at ~1000bp for the PFKA fragment), in the BamH1 and HindIII double digests, they were too faint to be seen on the gels. As the other digests (Ndel and XbaI/PvuII) did not provide conclusive evidence for a clone, we cannot, therefore, conclude that we have successfully ligated the desired cut pRSETA and cut PCR product fragments, or successfully amplified, purified, and genetically homogenized the inserted plasmid by transforming it into E. Coli DH5α bacteria. There were many failures or erroneous results in our experiment that need to be accounted for in this lab. While some of them are clear cut (like why our minipreps failed from the overnight ligation the first time – Figure 6) , others are more mysterious (like our first PCR – Figure 1).

Our second PCR (Figure 2) was carried out under identical conditions of temperature cycles, primers, chromosomal DNA, and magnesium concentration as our first PCR. Yet, our first PCR produced only a band of nonspecific DNA, while our second PCR produced the desired product. The most obvious explanations for this discrepancy is that our first PCR was missing a critical ingredient (out of inexperience), or that the thermal cycler was not working properly at the time.

Our first ligation (of the products of gel purification from Figure 3) was not successful (both in the sample transformed after 1.5 hours of ligating and in the sample ligated overnight) in that no bacteria transformed with it showed the PFKA insert in the pRSETA plasmid. While nearly all transformed colonies showed the pRSETA plasmid, none showed the insert. This was most likely due to a poor ligase enzyme, as others in the lab were having problems with ligations at the same time as I was. Another source of error could be that the pRSETA and PCR product did not cut properly in the pre-gel purification digest (as explained earlier, the gel purification gels of the PCRs– Figures 3, 8, 10, and 17 are not diagnostic to whether the PCR product was cut or not).

Our minipreps from colonies transformed with the 1.5 hour ligation (first try) did not extract but trace DNA from the bacteria. This was most likely due to the fact
that I tried to miniprep, digest, and run 36 samples of bacteria simultaneously. In the process of dealing with so many samples at once, I must have mis-timed important time-dependent processes, such as thorough vortexing of the sample after boiling, and the timing of the cooling and heating phases of the miniprep, as well as the restriction digest. Another, less likely source of error could have been that the miniprep lysis buffer I was using did not work properly, though others used tubes of buffer prepared from the same batch and had no problem.

Our retry of digesting and restriction analyzing minipreps from the colonies transformed with the 1.5 hour ligation (first try) contained large amounts of chromosomal DNA, as evidenced by the large bright spots right after the wells. This is certainly due to the fact that we had to realiquot the now frozen minipreps into the digest mixture. In the process of rethawing and realiquoting, the cellular debris and chromosomal DNA at the bottom of the tubes became agitated, and some of it resuspended into the supernatant. When we aliquoted the supernatant into the restriction digest tubes, it was therefore unavoidable to pick up some of the resuspended chromosomal-DNA.

In our second and third retries of the cloning procedure winter term, we eliminated several sources of error: the pure plasmid not cutting – Figure 14, too high DNA concentration in Trial 3, and impurities in the PCR samples preventing successful digestion and purification of the PCR product in PCRB2Pur in our second try. However, we were still unable to obtain a clone. In particular, the seeming success of our project up to the stage of ligation (as in the weird heavy fragment in Figure 11) and gel purifying the cut plasmid selectively (as in the double and triple bands seen in Figures 19 and 18 respectively) even at very low concentrations of pRSETA plasmid (1uL) and long digestions (8 hours), as well as the absence of a Pvull site on the miniprep DNA (Figure 23), and the confirmed presence of the working BamH1 and HindIII sites (Figure 14) suggests that somehow the bacteria or the ligase is modifying our neat cloning procedure and forcing “impossible” clones by religating non-complimentary sticky ends of the cut pRSETA without a Multiple Cloning Site (as evidenced by no Pvull site).

Another equally probable possibility for what is going on is that the cut pRSETA (without Pvull site) is religating with itself in pairs as evidenced by the minipreps with two bands (Figures 20 and 21 as evidenced by 2 bands of uncut dimer pRSETA and cut linear pRSETA identical to the gel purification product), as well as the ultraheavy band in the ligation (Figure 11), and some of the minipreps running heavy (indicating a 5.6kbp fragment dimer). However, some of the non-cutting single band minipreps (see Figures 4, 12, 21) point more towards the previous theory of single religations by unknown method instead of dimers. The most probable explanation for our results is that both processes, dimers and monomers of pRESTA being religated together – even despite of non-complimentary ends of the cut pRSETA without a Multiple Cloning Site (as evidenced by no Pvull site).

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this pRSETA dimer and religated gel purification product monomer minus the cut out Multiple Cloning Site.

One additional source for the failure of this experiment is that the presence of our cloned plasmid in the newly transformed bacteria is fatal. It is possible that even though the plasmid is specifically designed only to work with the T7 RNA Polymerase, that the gene for PFKA is being expressed from our plasmid by a similar RNA Polymerase or unknown mechanism, and that the resulting overproduction of PFKA is fatal or at least very negatively selective on the population of transformed cells. As PFKA is involved in a very vital part of cellular metabolism (glycolysis), it would not be surprising that if PFKA were overproduced in a cell, then that cell would might die due to its modified metabolism. In conclusion, the only way to unravel the mystery of what is going on in this experiment is to sequence the DNA at every step along the way, which is both very time consuming at a lab like the one I work in and very tedious. So I have decided to abandon this project in hopes of finding success in another project on bioinformatics in the spring term.

**Literature Cited:**

Swiss-Prot, <http://us.expasy.org/sprot> offers very specific information about certain proteins, such as inhibitors and activators. PFKA can be found by searching for P06998.

Invitrogen pRSET A, B and C online manual <http://www.invitrogen.com/content.cfm?pageid=7471> is a comprehensive manual explaining the properties and methods of proper use of the pRSETA plasmid we used in this lab.


Bio 600 supplement, 2002 contains most of the procedures, such as DNA miniprepping, restriction digests, gel electrophoresis, PCR, and plating and picking colonies.

Bio 560 supplement, for data on details and thermodynamics of glycolysis.
Previous Bio 600 papers contain the DNA purification procedure used on our E. Coli DH5α K12 chromosomal DNA.

**Appendix A:**

Sequence of PFKA gene (sequences complimentary to primers are highlighted, promoter highlighted, gene highlighted):

```
promoter 4711..4740
 NOTE= "factor Sigma70; predicted +1 start at 4105051"
 gene 4828..5790
 NOTE= "synonym: b3916"
 CDS 4828..5790
 NOTE= "gene pfkA"
 /EC_number="2.7.1.11"
 /function= "enzyme; Energy metabolism, carbon: Glycolysis"
 NOTE= "o320; 98 pct identical amino acid sequence and equal length to K6P1_ECOLI SW: P06998; CG Site No. 413"
 /codon_start=1
 /transl_table=11
 /product= "6-phosphofructokinase I"
 /protein_id="AAC76898.1"
/db_ref=GI:1790350"
/translation="MIKKIGVLTSGGDAPGMNAAIRGVRVRSALTEGLEVMGIYDYL 
LYEDRMVQLDRYSVSDMINRGGTFGLGSARFPEFRDENIRAVAIENLKKRGIDALVVIG 
GDGYSYMAGRTLTEMGFPCIGLPTIDNDKGTYDYGFTALSTVVVEAIDRLRTDSSS 
HQRRISYVEMGRYCDGLTLAAJAGGCCFVVVEPEFSREDLVNJEIKAGIAKGGKHAI 
VAITEHMCDVDELAHFIEKETGRETATVLAGHIOQGGSVPVPYDLRASRMGAYAIIDLL 
LAGYGGRCVGQNEQLVHHDIDIAENMKRPFKDWDLDCAKKLY"
```

4681 gtataaaata ccgccatttg gcctgacctg aatcaattca gcaggaagtg attgttatac
4741 tatttgcaca ttcgttggat cacttcgatg tgcaagaaga cttccggcaa cagatttcat
4801 tttgcattcc aaagttcaga ggtagtcatg attaagaaaa tcggtgtgtt gacaagcggc
4861 ggtgatgcgc caggcatgaa cgccgcaatt cgcggggttg ttcgttctgc gctgacagaa
4921 gctcgggaagt ttagggtat ttagaagce gcctggtgct ggtgaacgaa aacactgtaa
4981 cagtagatac gcctggtgct gcctggtgct cgcctggtgct gcctggtgct gcctggtgct
5041 cgctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct
5101 aacactgtaa gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct
5161 gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct
5221 gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct
5281 gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct
5341 gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct
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5461 gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct
5521 gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct
5581 gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct
5641 gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct
5701 gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct
5761 gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct
5821 gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct gcctggtgct
Sequence of pRSETA plasmid multiple cloning site and “tag”, starting with ATG (start codon) (restriction sites highlighted, His-tag highlighted) – also see the pRSETA manual <http://www.invitrogen.com/content.cfm?pageid=7471>:

Atgcgggttctcatcatcatcatcatggtatggtagcagactgtggacagcaaatgggtcgggatctgacgacgatgagcataaggatgatgagcatcgcagctgattaccatggaattcgaagcttgac

**Appendix B:**

The 16S Ribosomal RNA gene was used as a control on our second PCR, as this gene must be contained in all E. Coli bacteria chromosomal DNA. Ribosomal RNA (rRNA) is an important part of the ribosome. These two primers (27f ± rRNA 5’ and 1525r ± rRNA 3’) were shown to work in a previous experiment, however, they were very old when we used them, and did not work in our experiment. The sequences of the primers are as follows (M acts as an A or C, W acts as an A or T, R acts as an A or G when binding to the gene)

27f (rRNA 5’) 5’ **AGAGTTTGATCMTGGCTCAG** 3’

1525r (rRNA 3’) 5’ **AAGGAGGTGWTCARCC** 3’