

Dna research paper



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I. Introduction

Over the course of the last few weeks, we conducted experiments in which our goal was to successfully ligate EGFP cDNA into pET41a(+) in order to transform our recombinant expression plasmids into clones of E. coli. After growing these cultures on varieties of agar plates, we attempted to isolate our recombinant plasmid DNA and ran multiple tests in order to confirm that the EGFP insert worked.

GFP was first cloned in 1994 from the jellyfish *Aequorea victoria* (Prasher et al., 1992). This useful gene glows green under specific lights allowing us to see when and where proteins appear. The type of GFP we used in our lab was EGFP, which is a humanized version of GFP (Yang et al., 1996). Today, GFP has become one of the most widely used proteins in biochemistry and has sparked many new discoveries in gene expression and protein targeting.

In order to clone EGFP, we utilized multiple procedures of recombinant DNA technology. This technology allows scientist to produce DNA artificially by extracting DNA from two different sources and combining them into a single molecule. The first successful production was done in 1972 at Stanford University, which opened a new horizon in drug development and medical discoveries. Human insulin, which is used to help treat diabetes, was the first registered drug to use recombinant DNA technology (Johnson, 1983).

II. Methods

Ligating EGFP cDNA into pET41(a)+ plasmid

The goal of this experiment was to ligate EGFP into pET41(a) plasmid to create a recombinant expression plasmid.

We set up 5 different ligation reactions (2 ligations & 3 controls) using stock tubes containing DNA of 25 ng/ul cut pET41a(+) DNA, 7ng/ul EGFP insert DNA and 25 ng/ul uncut pET-41a(+)/EGFP recombinant plasmid DNA. Our first ligation (ligation #1) consisted of a 1: 1 molar ratio of pET-41(a) vector to EGFP insert so we used 2 ul of NcoI/NotI cut pET-41a(+) DNA, 1 ul of EGFP insert as well as 14 ul of sterile dH₂O, 2 ul of 10x ligase buffer and 1 ul of DNA ligase to make a final volume of 20 ul. Our second ligation (ligation #2) had a 1: 3 ratio of pet41a(+) and EGFP insert so we used 2 ul of Nco/NotI cut pET41a(+) DNA and 3 ul of EGFP insert as well as 12 ul dH₂O, 2 ul 10x ligase buffer and 1 ul DNA ligase. Ligation #3 was our positive control and we only used 2 ul of uncut pET-41a(+)/EGFP recombinant plasmid DNA. Ligation #4 and Ligation #5 were both negative control. In Ligation #4, only 20 ul of sterile dH₂O was used and in Ligation #5, 2 ul of Nco/NotI cut pET41a(+) DNA, 3 ul EGFP insert, 13 ul sterile dH₂O and 2 ul 10x ligase buffer were combined but no DNA ligase.

For each of our ligations, we mixed the components in microcentrifuge tubes and centrifuged each tube to bring all contents to the bottom of the tube. We then incubated the tubes for 10 minutes at room temperature. To prepare our samples for gel electrophoresis, we micropipetted 10 ul of each ligation as well as 2 ul of track dye into new, separate microcentrifuge tubes. In order to create a template to compare our results to, we also pipetted 12 ul of DNA ladder into a tube. We then loaded our 5 ligations and the DNA ladder

into a 40 ml 0.8% agarose gel at 60 V initially before turning the voltage up to 110 V in order to allow the gel to run faster.

Transformation of E. coli

The goal of this experiment was to transform our recombinant expression plasmid into a bacterial host, in this case E. coli.

We set up a total of 4 transformations, each in respect to ligations #1-4. 20 ul of competent E. coli cells was transported into four labeled and chilled microcentrifuge tubes. We then added 2 ul of each ligation mix into their appropriate tube of competent cells, mixed them together then immediately placed all the tubes on ice and incubated them for 5 minutes. The tubes were then heat shocked in a 42°C heat block for 2 minutes, and then immediately put back on ice for another 2 minutes. 80 ul of Luria brother (LB) was then added into each tube and put into a shaking incubator for 45 minutes at 37 °C.

While our transformations were incubating, we obtained 6 petri dishes. Four of the plates were LB/kanamycin/IPTG plates (labeled plates 1-4), one was a LB/kanamycin plate (labeled as K2 and also our negative control #1) and the last one was just a LB plate with no kanamycin or IPTG (labeled as K4). After 45 minutes of incubation, we began plating the cells onto each plate. 80 ul from each of ligations were plated onto their respected plates (1-4).

Additionally, 20 ul of Ligation #2 was plated onto K2 and 20 ul of Ligation #4 was plated onto K4. We then placed each inverted plate in a 37°C incubator and allowed them to grow overnight.

Miniprep of Plasmid DNA

The purpose of this experiment was to isolate plasmids from 3 different liquid cultures of E. coli

We were given three 3ml cultures of E. coli that were picked from the petri dishes we plated previously. The first culture contained a colony that fluoresced green under UV light and was the second contained a colony that did not fluoresce green under UV light. Both cultures were picked from one of our experimental ligations that were plated on our LB/Kan/IPTG plates. The third colony was picked from ligation #2 that was plated on the LB/Kan plate and was considered our “ unknown sample”.

For our miniprep experiment, we began by transferring 1.2 ml of each of our cultures into separate microcentrifuge tubes. We then pelleted the cells by centrifuging our tubes at max. speed for one minute and removed the supernatant with a P-1000 micropipette. Next we added 200 ul of Cell Suspension Solution to our tubes and vortexed each tube to disperse the cells in the solution. We then mixed in 200 ul of Cell Lysis Solution to each of our tubes and then added 200 ul of Neutralization Solution to create a precipitate. To pellet the precipitate we centrifuged our solutions for 5 minutes, then extracted the supernatant which would contain our DNA very carefully. For each of our minipreps, we prepared one Wizard Minicolumn by removing the plunger of a 3ml syringe and attaching the syringe barrel to the Luer-Lok ® extension of our labeled minicolumns.

Next we were given 1 ml tubes of resuspended DNA Purification Resin to mix into each of our preps where we then transferred 1 ml of the resin

suspension to the barrel of the syringe/minicolumn. To load the plasmid DNA into the barrel, we pipetted the supernatant out and transferred it into the barrel of the syringe containing the purification resin. We then pushed the DNA/resin mixture into the minicolumn, then disassembled our syringe/minicolumn properly before pipetting 2.0 ml of Column Wash Solution and pushing it through the resin of the minicolumn. Lastly, we dried the resin by transferring it onto a 1.5 ml microcentrifuge and centrifuging it for 2 minutes before finally transferring the solution into a final tube. After 50 μ l of TE buffer was added, we centrifuged our tubes for 20 seconds where they were then ready to be used directly as plasmid DNA.

Restriction Digest of Plasmid DNA

The purpose of this experiment was to run a variety of restriction digests (or none at all) through our isolated plasmids in order to determine the presence or absence of inserted plasmids for each of our clones.

We set up a total of two digests for each of our three plasmid DNAs isolated earlier from our miniprep, one with NotI/NcoI enzymes and one with just the NcoI enzyme for a complete total of six restriction digests. We were then given two "master mixes" containing the correct volumes of the reagents needed for either a single digests or a double digests and pipetted out 15 μ l of each master mix as well as 5 μ l of plasmid DNA into their appropriate tubes to get a total of 20 μ l for each of our digests. While incubating our digest at 37 °C for 1 hour, we prepared our "undigested" samples for each plasmid (3 total). Each undigested sample contained all the same ingredients at the same concentration as the digested samples except no enzyme was

added. This was all also given to us (besides the DNA) in a different master mix. We then pipetted out 15 ml of the undigested master mix into three microcentrifuge tubes and added 5 ml of DNA.

After being incubated at -20°C for one week, we ran our 9 digest samples (3 single digests, 3 double digests and 3 undigested) through a 50 ml 0.9% agarose gel at 60 V initially before turning it up to 110 V to allow the DNA to travel faster.

Polymerase Chain Reaction (PCR)

The purpose of this experiment was to properly set up and run polymerase chain reactions for our recombinant plasmids using specified primers to confirm the identity of our plasmids.

The primers we used, pAD1sense and pAD1anti were specific for our pET41-EGFP recombinant plasmids. The DNA templates we used were the three plasmids we isolated earlier in our miniprep, a given positive control of pET41/EGFP recombinant plasmid as well as a negative control, which contained no DNA. After being given our stock reagents, in order to form our own master mix we calculated the appropriate values for each reagent to make enough mix for a total of 20 ul for each PCR (71.4 ul nuclease-free H₂O, 12 ul 1X PCR buffer, 9.6 ul 800 uM dNTPs, 6 ul 0.5 uM pAD1sense primer, 6 ul uM pAD1anti primer and 3 ul 2.5 units Taq polymerase). We then pipetted out 18 ul of master mix into each of our five tubes as well as 2 ul of each appropriate DNA template. We then incubated our samples in the thermocycler in the following parameters:

95°C for 10 mins

95°C for 1 min

56°C for 1 min

72°C for 1.5 min

4°C (hold, to suspend reaction/avoid degradation of DNA)

We then ran our 5 DNA samples through a 40 ml 0.9% agarose gel at 60 V before turning it up to 110 V to allow our DNAs to run faster.

Virual Cloning and Sequence Analysis

The purpose of this lab was to use online resources to determine the nucleotide sequence of our EGFP/pET41 recombinant plasmid, the primer locations and size of the PCR produce and to design a restriction digest experiment to future prove the identity of our expected DNA sequence.

To do this procedure, we used a variety of online tools. First, we used a nucleotide database to find the pEGFP-N1 plasmid nucleotide sequence. Next we used the WatCut restriction analysis tool to find the cut sites we used to cut the EGFP fragment out of the pEGFP-N1. To find the pET41(1) vector sequence, we used the LabLifeVector Database. Based on all the information and sequences we found from these databases, we then virtually “ligated” our EGFP fragment into the cloning site of the pET41(+) plasmid sequence to generate our entire recombinant plasmid sequence. From here, we searched for the location of our two primers, pAD1sense and pAD1anti, to determine the size of the PCR product.

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To further confirm the identity of our product, we designed a restriction enzyme digest experiment using WatCut and two different enzymes that would cut the PCR products appropriately and determined the sizes of the outcome of fragments.