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A multisession, undergraduate molecular biology lab experiment using Green Fluorescent Protein including subcloning and color changing mutagenesis.

by Nathan Winter
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Abstract This paper describes a series of experiments involving handling and manipulating the DNA coding for Green Fluorescent Protein (GFP) including the subcloning of this gene, and mutating the DNA so that Cyan Fluorescent Protein (CFP) or Blue Fluorescent protein (BFP) are expressed. The primers needed for the PCR based subcloning of GFP are presented, as are those needed to mutate the GFP to either CFP or BFP.

Introduction The experiments described below address many of fundamental, molecular biology experiments which have been developed and modified at Saint Cloud State University since 2012. These experiments introduce students to many basic techniques. There are two end results for the DNA manipulation portion of the lab: the subcloning of the GFP gene and the mutation of the GFP gene so that the protein fluoresces in a different color. The tyrosine at amino acid 66 is the residue which is modified. If this amino acid is changed to histidine, BFP is produced. CFP is made when this amino acid is tryptophan[1].

The experiments that we do weekly during our class, Biochemistry II, (which has a emphasis in molecular biology) are listed below.

1. Reagent preparation and pouring plates.
2. Running a transformation experiment using pBAD-GFPuv (pGLO™) from Biorad.
3. Harvesting the pBAD-GFPuv using a silica spin column.
4. Polymerase chain reaction (PCR) of the Green Fluorescent Protein gene.
5. Agarose gel electrophoresis to isolate the PCR product.
6. Gel purification of the PCR product using a silica spin column.
7. Subcloning of the PCR product into a blunt end vector.
8. PCR based site directed mutagenesis of pBAD-GFPuv so that the DNA expresses Cyan Fluorescent Protein or Blue Fluorescent Protein.
9. Expression and purification of the Fluorescent Proteins by hydrophobic interaction chromatography.

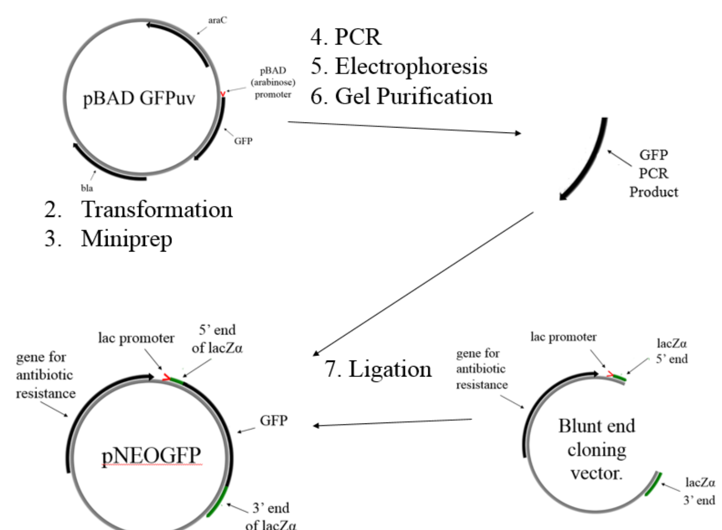

Figure 1 Subcloning of GFP This figure uses the same numbering scheme as the list of weekly experiments shown above. When in pBAD-GFPuv the GFP is induced by arabinose, but because the PCR primers do not include the pBAD promotor, when subcloned into a blunt end vector, the gene is induced by IPTG.

The Experiments

1. Reagent Preparation and Pouring Plates. The first lab period is used as an opportunity to introduce or review aseptic techniques. In addition, reagents needed periodically throughout the semester are made, including one mL aliquots of sterile filtered, 40 mg/mL sodium salt of ampicillin, 10 mL aliquots of sterile filtered, 200 mg/mL L-arabinose, and about 25 mL sterile, 30% glycerol. Each student pair makes 10 mL of sterile LB in a culture tube, and pours four LB-agar plates: one with no supplements, two with ampicillin and one with 50 ug/mL ampicillin and 6 mg/mL L-arabinose.

> **Protocol for pouring the four plates.** To prepare the four LB-agar plates the students sterilize 80 mL of LB-agar, and label the four 100 mm Petri dishes. When the hot flask of LB-agar has cooled enough to hold in the palm of the hand, the first plate is poured with no supplements. Then stock ampicillin is added to the remaining ~60 mL of LB-agar so that it is 50 ug/mL. The two LB-agar/amp plates are then poured. Filter sterilized arabinose is added to the remaining ~20 mL LB-agar with amp to make the solution 6 mg/mL L-arabinose and the final plate is poured. Additional plates will need to be made as needed during the semester.

2. Transformation with pBAD-GFPuv. The plasmid and bacteria needed for this lab are found in the pGLO™ Bacterial Transformation kit from Bio-Rad. Transformation procedures provided in the kit work well. The kit has enough plasmid DNA for about twenty student pairs. Plates with successful transformants are sealed with a paraffin film and stored at 4° C.

3. Plasmid Miniprep. The evening before the lab period, the students supplement the 10 mL of LB with ampicillin to 50 ug/mL and inoculate with a colony from their plate of successful transformants. The cultures are grown overnight is a shaking incubator. During the lab period on the next day they use the E.Z.N.A Plasmid Mini Kit I, a silica based spin chromatography column, to harvest the plasmid DNA using the procedure associated with the kit.

I also have each student pair make a glycerol stock of their overnight culture by vortex mixing 500 uL of their overnight and 500 uL of sterile 30% glycerol in a sterile cryovial, and then flash freezing in liquid nitrogen. The stocks are stored in a -80° freezer to have the plasmid containing cells available if needed.

4. PCR of the GFP gene. The GFP gene from pBAD-GFPuv was amplified using PCR. We have used Go*Taq*™ Green Master Mix from Promega following the vendor provided instructions. We used the primers shown in figure 2 which were ordered from Integrated DNA Technologies.

I designed our primers using the on-line software, primer 3[1]. The left primer had to be designed to meet a number of criteria. First the PCR product had to be in phase with the lac promoter of the accepting vector. Second the primer could NOT in contain the stop codon six triplets upstream from the GFPs initiating methionine; this would result a plasmid in which the translation from the lac promoter would stop before reaching the GFP gene. In order to avoid the this stop codon, I searched for primers farther and farther to the right. So far downstream, that some of the final constructs would not express the N-terminus amino acids from GFP; however, these truncated proteins will fluoresce as long as they contain the tandem glutamates at position 6 and 7[2].

Subcloning Primers	Sequences	Nucleotides	Tm
Left	aaa gga gaa gaa ctt ttc act gga	24	59.8
Right	ctt ctc tca tcc gcc aaa ac	20	59.8

```
        Stp                          MetAlaSer LysGlyGluG luLLeuPheThrGlyValVal
1321 ctttaagaag gagatataca tatggctagc aaaggagaag aacttttcac tggagttgtc
                                      >>>>>>>>>> >>>>>>>>>> >>>>


     MetAspGluL euTyrLysSt p
2041 atggatgagc tctacaaata atgaattcga gctcggtacc cggggatcct ctagagtcga

                                 <<<<<<< <<<<<<<<<< <<<
2101 cctgcaggca tgcaagcttg gctgttttgg cggatgagag aagattttca gcctgataca
```

Figure 2 The subcloning PCR primers. In addition to the two primers, their binding sites on pBAD-GFPuv are also shown. The entire GFP genes extend from nucleotide 1342 to 2061, and only a portion of this is displayed above. The primer binding sites are marked with > or <. Protein coding regions are marked with the amino acids for which the nucleic acid triplets code. Note that the left primer does not contain the stop codon at nucleotides 1324-1325-1326. Rather it starts at nucleotide 1351. So the first three amino acids of the starting GFP will not be present in the GFP produced in the subclone. All the nucleic acid sequences are listed in the 5' to 3' direction.

5. Agarose gel electrophoresis to isolate the PCR product. The students use agarose electrophoresis with 1% gels to isolate their DNA. 3 uL of Sybr™ Safe Stain was added to the liquid agarose before pouring. Molecular weight standards did not need to be run because the lanes have only one sample, the PCR product. In addition, GoTaq Green Master Mix contains a yellow dye which migrates at nearly the same distance (~500 bp) as the PCR product (801 bp). The gels were visualized using an inexpensive, blue light, LED Transilluminator from IORodeo. The bands containing the PCR product were cut out and frozen.

6. Recovery of the PCR product by silica spin chromatography. The student ran the E.Z.N.A.™ kit to obtain their PCR product.

7. Subcloning of the PCR product into a blunt end vector. To complete the subcloning project, the students use a kit which come provided with the blunt end vector and chemically competent cells. Following the kits instructions, the students ligate their PCR product into the blunt end vector, and then use their new plasmid to transform cells. If their subcloning is successful, the transformed cells will glow green. We have successfully used a commercially available, blunt end cloning vector to complete this project; however, because they no longer provide their chemically competent cells in a single use volumes our success with transformation dropped markedly, and I cannot make a recommendation for a blunt end, cloning kit at this time. Anyone adopting this protocol needs to ensure that their primers will make a PCR product which is in phase the promoter provided by their vector.

8. PCR based site directed mutagenesis of pBAD-GFPuv so that the DNA expresses Cyan Fluorescent Protein or Blue Fluorescent Protein.

Different colored fluorescent proteins are made by changing a single amino acid of GFP, tyrosine 66[1]. If this amino is changed to histidine, BFP is made. A tryptophan substitution will makes CFP. In order to make these changes, a TAT codon has to be changed to either CAT or TGG, for BFP and CFP respectively. You should be aware, that of the two mutations the CFP is better. CFP's quantum yield of the fluorescence is around 0.40, where the BFP has a dim quantum yield of only 0.24. For comparison, GFP has a quantum yield of 0.79, significantly brighter.

To make these mutations the students use Q5 Site-Directed Mutagenesis Kit from New England Biolabs using primers designed online using NEBaseChanger. These primers and are shown below with the mismatched bases (the mutagenic bases) highlighted. We have had good success following the PCR and transformation procedures given with this kit.

Primer	Sequence	Length	Tm	Ta
BFP forward	5′ TAC TTT CTC T**c**A TGG TGT TCA ATG C 3′	25	58° C	61° C
BFP reverse	5′ GTG ACA AGT GTT GGC CAT G 3′	19	62° C	
CFP forward	5′ ACT TTC TCT T**gg** GGT GTT CAA TGC TTT TC	29	58° C	61° C
CFP reverse	5′ AGT GAC AAG TGT TGG CCA TG 3′	20	63° C	

9. Purification of fluorescent proteins by Hydrophobic Interaction Chromatography. We use a modification of procedure by David Micklos.[4] The night before the lab period, each student pair inoculate one culture tube containing 10 mL of LB with either GFP, BFP or CFP. The broth needs to be supplemented with ampicillin to a final concentration of 50 ug/mL and the appropriate inducer: either IPTG to 0.5 mM concentration or L-arabinose to a 6 mg/mL concentration.

The next day, 1 mL of the overnight is transferred into each of two microcentrifuge tubes. The cells are pelleted in a microcentrifuge for 5 minutes at the maximum speed, and the supernatant is poured off. This procedure is repeated 3 more times so the 4 mL of the culture have contributed to each pellet. The pellets are suspended in 250 uL of TE buffer (all the TE buffers used in this procedure are pH 8). 100 uL of 20 mg/mL lysozyme is added to each tube, and the mixtures are incubated at 37° C for an hour. The tubes are

then frozen to break the cells with ice crystals. This freezing can be overnight, but if time is limited, liquid nitrogen can be used to flash freeze. The microcentrifuge tubes are thawed, and then centrifuged for 5 minutes at 6000 x g. 250 uL of the supernatant is taken from the two tubes, and transferred to a single microcentrifuge tube, making 500 uL. To this 250 uL of 4 M ammonium sulfate in TE buffer are added. Store the sample at 4° C until applied to the chromatography column.

For the chromatography matrix, we use Macro-Prep Methyl HIC media from Bio-Rad. The storage solution over the media is removed without disturbing the media, and an equal amount of 2 M ammonium sulfate in TE buffer is added. The bottle is rocked to suspend the HIC media. Less than three mLs of the media is added to a narrow chromatography column to a height of about 2 cm. For our columns we use medicine droppers with a bit of glass wool to prevent the stationary phase from washing out, with tubing and pinch clamp over the bottom end to control the flow rate. After packing the matrix is washed with 1 mL of 2 M ammonium sulfate in TE buffer, the buffer is allowed to flow through until the top of the matrix is covered, and the protein solution can then be applied. At this time we start collecting 0.5 mL fractions. Once most of the protein solution is in the matrix, add a small amount of 2 M ammonium sulfate in TE buffer, and let that flow into the column. Now add 1000 uL of 1.3 M ammonium sulfate in TE buffer to the column, and when most of that is in the column, you can add TE buffer to elute the protein. 1500 uL should be enough. The fraction can be examine under UV light to detect the fluorescent protein.

The fluorescent proteins can then be further analyzed of course. The students could undertake fluorescent studies, run the proteins out on native polyacrylamide gels and so on.

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