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Use of the green fluorescent protein as an educational tool

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The green fluorescent protein (GFP) is a bioluminescent protein that can be expressed and easily detected as a fully fluorescent protein in both bacterial and eukaryotic cells. These properties, along with its ability to withstand exposure to denaturants, organic solvents, high temperature and a wide pH range, make GFP an ideal educational tool. To that end, two GFP-based laboratory modules are described that can be used to teach recombinant DNA and protein purification techniques to high school and undergraduate college students. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 323–326.

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Introduction

The green fluorescent protein (GFP), from the jellyfish *Aequorea victoria*, is bioluminescent and absorbs blue light at 395 nm and emits brilliant green light at 509 nm [12]. Unlike other bioluminescent proteins, GFP can be expressed as a fully fluorescent protein in a variety of heterologous cell types including animals, plants, yeast, and bacteria with no apparent substrate or cofactor requirements [1,3]. This property has led to the widespread use of the GFP cDNA as a fluorescent marker for gene expression and as a tool to study protein–protein interactions [1,3,5,7]. Over the past few years, several red- and blue-shifted spectral mutants of GFP, yellow fluorescent protein (YFP). and blue fluorescent protein (BFP) have been created, and like wild-type GFP, have been used extensively as reporter genes [3,4].

In contrast with other bioluminescent proteins, such as bacterial luciferase and aequorin, GFP and its color variants are unusually stable proteins. Ward and colleagues determined that GFP can withstand exposure to high temperature (65°C), organic solvents (50% ethanol), denaturants (8 M urea), preservatives (formaldehyde) and several proteases [8,9,13,14]. Further, GFP can be stored at room temperature for up to a year without substantial loss of fluorescence. Most important, GFP and its color variant, BFP, can easily be detected following exposure to long-wave ultraviolet light (LWUV) from a handheld lamp.

The unprecedented growth of the biotechnology industry in the 1990s has prompted many high schools and universities to offer degree and training programs in biotechnology. This, in turn, has forced science educators to search for innovative pedagogical tools to help students master the underlying principles of molecular biology and biotechnology. GFP's brilliant green color and its ease of detection, coupled with its unusual stability and the availability of color variants, suggest that GFP would be an ideal teaching tool. To that end, two GFP-based laboratory modules were designed to teach high school and undergraduate college students some of the salient features of recombinant DNA technology and protein purification.

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Materials and methods

Plasmids and molecular biology reagents

pBAD-GFPuv (ClonTech, Palo Alto, CA, USA; Figure 1) is an expression vector that carries the Stemmer cycle 3 GFP mutant ([2], Table 1) under control of the positively-regulated arabinose operon from *Escherichia coli*. pBAD-BFP was constructed by deleting the *Nde*I-*Eco*RI fragment that contained Stemmer cycle 3 GFP gene from pBAD-GFPuv and inserting an *ca* 1.2-kb *Nde*I-*Eco*RI fragment containing the blue-shifted BFP gene (Table 1) in its place (downstream of the arabinose promoter).

The plasmid, pHisflexGFP, was constructed by 'add on' polymerase chain reaction (PCR) using pBAD-GFPuv as

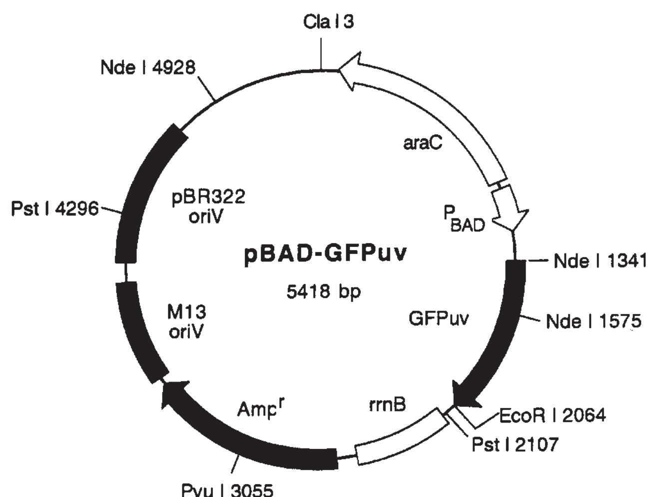

Figure 1 Restriction map of pBAD-GFPuv. GFPuv (Stemmer cycle 3) is under control of the arabinose promoter from *E. coli*. Arrowheads indicate the direction of transcription. Numbers represent the position of available restriction sites for mapping experiments. Abbreviations: Amp^r, ampicillin resistance; OriV, origin of vegetative replication; rrn, transcription terminator.

Table 1 Spectral properties of GFP and its color variants

Protein	Absorption (nm)	Emission (nm)	Color	Mutation(s)
GFP	392	509	green	none
Stemmer cycle 3	392	509	green	F100→S, M154→T, V164→A
BFP	382	448	blue	Y66→H
YFP	513	527	yellow-green	S65→G, V68→L, S72→V, T203→Y

template DNA [11]. Briefly, primers were designed to add a flexible linker(ser-$(gly)_3$) followed by six histidine residues to the 3′ end of the Stemmer cycle 3 GFP gene in pBAD-GFPuv. Inclusion of a flexible linker in recombinant his-tagged GFP improved purification by metal chelation chromatography. Also, a *Kpn*I site was introduced immediately 5′ of the flexible linker to provide a convenient restriction site for additional cloning experiments.

Restriction enzymes, DNA ligase, and other molecular biology reagents were obtained from New England BioLabs (Beverly, MA, USA) or Promega (Madison, WI, USA). Ampicillin (Ap) and arabinose were obtained from Sigma Chemical Company (St Louis, MO, USA).

Transformation and plasmid isolation

$CaCl_2$-treated cells of *E. coli* DH5α were transformed with GFP- or BFP-containing plasmids using standard methods [6]. Fluorescent green or blue colonies were identified on LB agar containing 100 μg per ml of Ap and 0.2% arabinose (w/v) after 24–48 h at 37°C using a handheld LWUV lamp (Bio-Rad Laboratories, Richmond, CA, USA). Individual transformants were picked from the selection plates, inoculated into 8 ml of LB plus Ap and arabinose, and grown overnight at 37°C. After incubation, bacteria were collected from 2.0 ml of overnight cultures via centrifugation in a microfuge and plasmid DNA was isolated using Qiagen plasmid isolation kits (Qiagen, Valencia, CA, USA). Plasmid DNA was digested with appropriate restriction enzymes and the resultant digests were analyzed by agarose gel electrophoresis using 0.8% agarose gels. DNA restriction fragment patterns were visualized by ethidium bromide staining.

Purification of recombinant GFP and BFP

Bacteria harboring pBAD-BFP and pBAD-GFPuv were used to obtain recombinant GFP (rGFP) and BFP (rBFP). Briefly, rGFP and rBFP were purified from cell lysates by gel permeation chromatography, followed by ammonium sulfate precipitation and anion exchange chromatography [10]. Fractions containing either GFP or BFP were pooled and stored at 4°C in 0.1% sodium azide.

Isolation of his-tagged GFP

E. coli DH5α that harbored pHisflex GFP which encodes his-tagged GFP (htGFP) were grown at 37°C in 50 ml of LB broth supplemented with Ap (100 μg per ml) and 0.2% arabinose (w/v). After 16 h, bacteria were harvested by centrifugation at $8000 \times g$ for 20 min and the resultant cell pellet was lysed by sonication (one 10–15 s burst at high setting) to release cytoplasmic htGFP. Immediately following sonication, the cell lysate was suspended in 15 ml of 10 mM Tris-HCl pH 8.0 (to inhibit bacterial proteases) and 5.0 ml of the lysate was loaded onto a Quiagen NTA nickel chelation chromatography column (Quiagen). The column was washed twice with 5.0 ml of 10 mM imidazole in 10 mM Tris-HCl pH 8.0 and htGFP was eluted from the column by addition of 5.0 ml of 0.6 M imidazole in 10 mM Tris-HCl pH 8.0. Fractions containing htGFP were identified using a handheld LWUV lamp.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a standard Laemlli buffer system. Molecular size markers (Bio-Rad) and samples for PAGE were boiled for 1 min in sample buffer (63 mM Tris-HCl pH.6.8, 10% glycerol, 2% SDS) and then separated on a 12% running gel (3.0% stacking gel) using a Bio-Rad Mini-Protein gel box (Bio-Rad). The running buffer used in these experiments was 25 mM Tris-HCl, pH 8.3, 192 mM glycine and 0.1% SDS. Samples were electrophoresed for *ca* 90 min at a constant voltage of 125 V. After electrophoresis, proteins were visualized by staining gels with Coomassie R250. Non-denaturing (native) gel electrophoresis (ND-PAGE) was carried out as described above except that there was no SDS in the sample and running buffers. GFP and BFP were visualized in ND-PAGE gels using a handheld LWUV lamp.

Results and discussion

Recombinant DNA techniques module

The objectives of the recombinant DNA techniques module were to familiarize students with bacterial transformation, plasmid isolation, agarose gel electrophoresis, restriction endonuclease analysis and plasmid mapping. The experiments in this module were typically completed in four to five 2.5-h laboratory sessions.

In the first set of experiments, $CaCl_2$-treated *E. coli* DH5α cells were transformed with pBAD-GFPuv or pBAD-BFP and plated for single colonies on LB agar that contained Ap for selection and arabinose to induce expression of GFP or BFP. After 24–48 h of incubation at 37°C, transformants were identified as fluorescent green or blue colonies following exposure to LWUV light. In subsequent experiments, plasmid DNA was isolated from transformants expressing either GFP or BFP, digested with appropriate restriction enzymes (see Figure 1), and subjected to agarose gel electrophoresis. Molecular size markers (lambda-*Hin*dIII ladder) were included on all gels. After electrophoresis, agarose gels were stained with ethidium bromide to visualize DNA fragments. The restriction fragment patterns resulting from digestion of plasmid DNA with one, or combinations of two or more restriction enzymes permitted students to construct rudimentary restriction maps of pBAD-GFPuv or pBAD-BFP.

Although the recombinant DNA techniques module was

well received by students, several minor changes will be made to improve the delivery and overall effectiveness of the module. First, the use of pre-poured agarose gels (FMC, Portland, ME, USA) should eliminate technical problems and time constraints that typically result when students pour their own agarose gels. Second, using commercially available non-toxic DNA stains should eliminate safety concerns about the use of the mutagen, ethidium bromide, to stain DNA. Third, the use of commercial purification kits to isolate plasmid DNA reduces the time required for pre-laboratory preparation and insures isolation of 'quality' plasmid DNA. Finally, we are re-engineering pBAD-GFPuv and pBAD-BFP to introduce more convenient restriction enzyme sites for plasmid mapping experiments.

Protein purification module

The goals of the experiments in the protein purification module were to introduce students to his-tag protein purification and polyacrylamide gel electrophoresis. Both techniques are commonly used for protein purification and analysis in the biotechnology industry. The experiments described in this module were typically completed in three to four 2.5-h laboratory sessions.

The addition of a His-Tag to the amino- or carboxy-terminus of a protein (using PCR or by cloning the gene into a His-Tag expression vector) permits a rapid one-step purification of the target protein by metal chelation chromatography [10]. The His-Tag sequence binds to divalent cations (Ni^{2+}) immobilized on the column matrix. After unbound proteins are washed away, the target protein can be recovered from the column by elution with imidazole.

In the first experiment, *E. coli* harboring pHisflexGFP (which encodes htGFP) were grown overnight at 37°C in LB broth plus Ap that contained arabinose to induce expression of htGFP. After incubation, bacteria were harvested by centrifugation and lysed by sonication to release htGFP from the cells. Immediately following sonication, the cell lysate was suspended in 10 mM Tris-HCl pH 8.0 and *ca* 5.0 ml of the htGFP-containing lysate was loaded on to a nickel column. The column was washed twice with wash buffer and then examined with a handheld LWUV lamp. This revealed that htGFP was retained on the nickel column as a tight green fluorescent band. Students were able to visualize and follow the removal of htGFP from the nickel column (after addition of elution buffer) by using a handheld LWUV lamp.

To demonstrate the principles of PAGE, rGFP-containing cell lysates and purified rGFP and rBFP were electrophoresed in 12% ND-PAGE and SDS-PAGE gels. After electrophoresis, proteins were visualized in SDS-PAGE gels by Coomassie blue staining (Figure 2a). rGFP was identified in whole cell lysates (lane 2) based on its co-migration with a 27-kDa band that corresponded to purified GFP (lane 3). In contrast, rGFP and rBFP were easily identified without staining on ND-PAGE gels upon exposure to LWUV. As shown in Figure 2b, rGFP could be identified in cell lysates as a single green fluorescent band (lane 1) that co-migrated with purified rGFP (lane 2) whereas purified BFP appeared as a single blue fluorescent band (lane 3) in ND-PAGE gels.

The experiments contained in the protein purification module were enthusiastically received by students. The his-tag protein purification experiments are straightforward and easy to perform. In contrast, the PAGE experiments are labor-intensive and require substantial pre-laboratory preparation. Not surprisingly, casting of PAGE gels proved to be challenging to many students. Consequently, we plan to use pre-cast PAGE gels in the electrophoresis experiments when this module is offered again. Also, we were unable to adequately 'fix' GFP and BFP in ND-PAGE gels. This forced students to examine ND-PAGE gels immediately after the experiment was completed. We are currently formulating a fixing solution that will preserve the fluorescent properties of GFP and at the same time prevent diffusion of the protein from ND-PAGE gels.

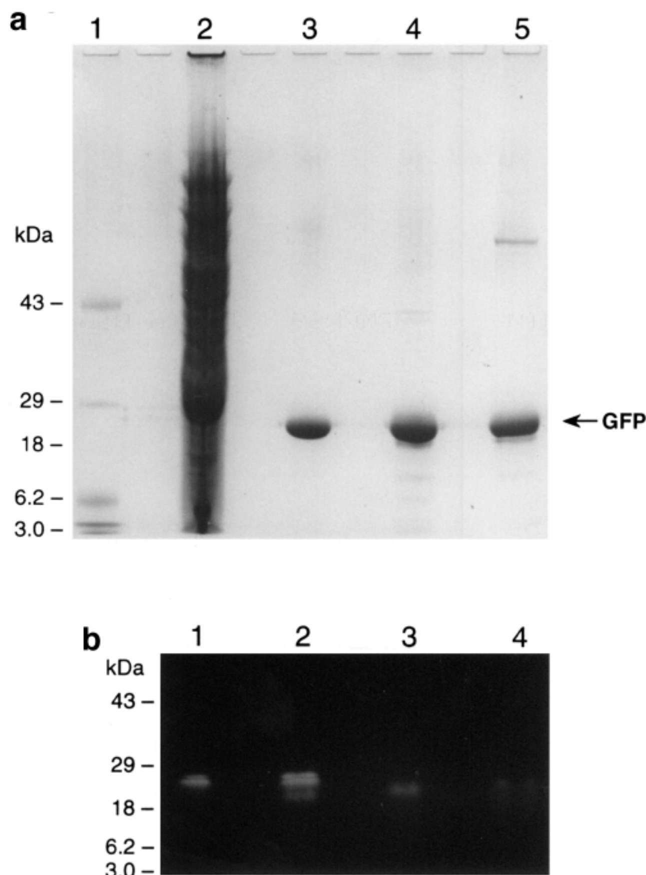

Figure 2 SDS-PAGE and ND-PAGE of GFP and BFP. (a) Coomassie blue-stained SDS-PAGE gel. Lane 1, molecular size markers; lane 2, cell extract containing rGFP; lane 3, purified rGFP; lane 4, purified rBFP; lane 5, mixture (1:1) of purified rGFP and rBFP. (b) ND-PAGE gel exposed to LWUV. Lane 1, cell extract containing rGFP; lane 2, purified rGFP; lane 3, purified rBFP; lane 4, mixture (1:1) of purified rGFP and rBFP. ND-PAGE gel was photographed using a UV-light box and a blue filter.

Concluding remarks

GFP has become popular in science education circles. It is becoming apparent to science educators, at all levels, that GFP is a very versatile and extraordinary teaching tool. Accordingly, in the present study, we used GFP and its color variants to create 'hands-on' inquiry-based teaching exercises that showcase principles and workplace practices

of the biotechnology industry. The low cost and modular design of our GFP-based exercises makes them ideal for use in high school, undergraduate college and continuing education biotechnology laboratory courses.

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