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A series of laboratory exercises utilizing *luxR* gene of *Vibrio fischeri* and *gfp* gene of *Aequoria victoria* to teach the broad applications of polymerase chain reaction

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Three experiments are described; directional cloning of the *luxR* gene from the bioluminescent marine bacterium, *Vibrio fischeri*, directional cloning of the *gfp* gene from the marine jelly fish, *Aequoria victoria*, and the construction of a LuxR-GFP fusion protein. Experiments are presented using *lux* and *gfp* in an undergraduate biology curriculum. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 345–352.

Keywords: polymerase chain reaction (PCR); *lux* bioluminescence; green fluorescence protein (GFP); directional cloning; fusion protein

Introduction

Polymerase chain reaction (PCR) is a powerful tool in the arsenal of recombinant DNA techniques. PCR is an *in vitro* reaction that allows a specific sequence of DNA to be amplified into millions of copies within a few hours. The technique involves the selection of two different primers (oligonucleotides) that are complementary to the two ends of the target DNA sequence [2,13]. Laboratory exercises are needed to teach this technology to students. Many PCR experiments amplify only a segment of DNA and cloning experiments involve only cloning and expression of an antibiotic resistance gene. In such cloning experiments, students begin with *E. coli* colonies on an agar plate and end with identical looking colonies. While these experiments are successful and instructive, they lack the wonderment that is the essence of genetic engineering. When PCR is combined with the *lux* bioluminescence and/or the green fluorescence protein (*gfp*) systems, students perform powerful and exciting experiments. The specific experiments described in this article include directional cloning of the *luxR* gene, directional cloning of the *gfp* gene, and construction of LuxR-GFP fusion protein. These advanced techniques are suited for a college level molecular biology course.

The lux bioluminescence system

In 1990, Slock [15] identified two regulatory domains of the LuxR protein, a protein that regulates bacterial bioluminescence. This research supported a hypothesis that some bacterial species can communicate and coordinate their activities to achieve a common goal. This system of gene regulation was termed quorum sensing [7–9] and to date more than 30 Gram-negative bacteria have been identified with homologous quorum sensing systems. Many of these bacteria enter into symbiotic or parasitic relationships with a plant or animal host.

The *lux* bioluminescence genes are the best understood quorum sensing system. They are found in the marine bacterium, *Vibrio fischeri*, in which the genes responsible for bioluminescence (*lux* genes) are expressed in response to increasing cell density. *V. fischeri* occurs at very high densities in specialized light-emitting organs of certain marine fish and squid where the light produced by the bacteria is used by the animal host. *V. fischeri* is also found at lower densities in seawater where it exists as a member of the bacterioplankton. In the planktonic habitat, light production is not important and would consume energy. The quorum sensing system enables *V. fischeri* to produce light in the symbiotic state (the light organs of fish) but does not allow light production in the planktonic habitat. The quorum sensing system of *V. fischeri* employs two genes, *luxI* whose gene product is responsible for the synthesis of a freely diffusible signal (an acyl homoserine lactone termed AI) and *luxR* whose gene product when complexed with AI activates expression of the *lux* structural genes [11].

A 9-kilobase fragment of *V. fischeri* DNA that encodes all of the functions for luminescence and contains regulatory elements sufficient for their expression in *E. coli* has been isolated. Seven *lux* genes have been defined and are organized as two divergently transcribed units shown by arrows in Figure 1. There are two other genes (*luxG* and *luxH*) located at the end of *luxE* but their functions are unknown. The region between the *luxR* and *luxI* arrows is the control region. The control region contains promoter and operator nucleotide sequences and thus is the place where RNA polymerase and regulator proteins bind. The left transcriptional unit contains the *luxR* gene which encodes the regulator protein (LuxR protein) required for cells to respond to the autoinducer (AI). The other unit contains *luxA* and *luxB*, which encode the α and β subunits of luciferase; *luxC*, *luxD*, and *luxE*, which encode proteins involved in synthesis of the aldehyde substrate for luciferase; and *luxI* which is the only *V. fischeri* gene required for synthesis of the autoinducer in *E. coli*.

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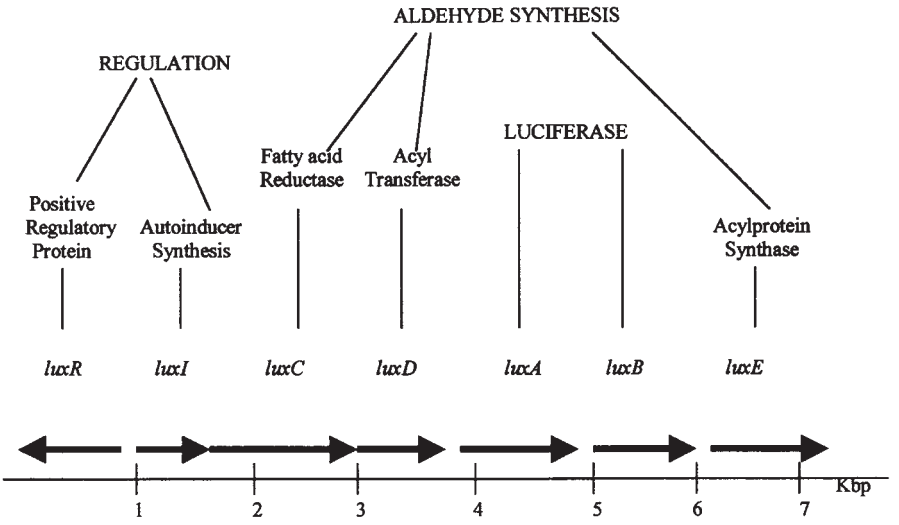

Figure 1 Organization of *V. fischeri lux* genes. The *luxR* regulon and *luxICDABE* regulon are transcribed divergently. The primary regulatory region resides between the *luxR* and *luxI* genes. Reprinted with permission of TO Baldwin.

The seven *lux* genes from *V. fischeri* have been cloned into two plasmids. Plasmid pHK724 [14] contains the *luxR* gene and plasmid pHK555 [14] contains the structural genes (*luxICDABE*) required to produce light. The *luxR* gene of pHK555 is inactive because of the insertion of phage DNA. When pHK724 is transformed into *E. coli* containing pHK555, the resultant colonies that grow on selective media bioluminesce [10,14,15].

Bacterial transformation experiments utilizing *lux* genes have been developed by Slock [14], Carolina Biotechnology [3] and Fotodyne, Inc [6]. Winfrey [17] has written a laboratory manual using *lux* genes for a series of experiments including DNA isolation, cloning, restriction mapping, Southern blotting and PCR.

The gfp fluorescence system

GFP is a protein isolated from the bioluminescent jelly fish, *Aequoria victoria*. GFP functions as an accessory emitter protein that shifts *A. victoria* bioluminescence from blue to green [4]. A protein extract of the bioluminescent tissue fluoresces green when excited by long-wave UV light. GFP remained in relative obscurity until the early 1990s when it was cloned [12] and expressed [5] in other organisms, including *E. coli*. The unique three-dimensional structure of GFP consists of a barrel structure (11 β-sheets) enclosing the chromophore. The chromophore forms by autocatalysis of three amino acids, and is suspended in the middle of the barrel by an α-helix coil [4]. Genetically engineered GFPs exist that have enhanced fluorescence and can be expressed in a variety of organisms. When the *gfp* gene is cloned into the genome of an organism, the resultant GFP-protein fusion can function as a reporter molecule for gene expression or track protein movement inside a living cell. Cells, and whole organisms, can be made to fluoresce green with a hand-held UV lamp. Subcellular organelles can be visualized with the use of a fluorescent microscope.

Bio-Rad Laboratory's Life Science Group [1] has developed an inquiry-based biotechnology curriculum with GFP as its cornerstone. The kits present real life, problem-centered scenarios. The three kits involving GFP are bacterial transformation, protein purification and secrets of the rain forest. Carolina Biotechnology also offers a bacterial transformation kit [3]. Green Fluorescent Protein [4] is a useful reference on all aspects of GFP.

Materials and methods

E. coli strains: *E. coli* Top 10 available from Invitrogen (www.invitrogen.com). *E. coli* (pHK555) obtained from author.

Preparations of competent cells and transformation: Described in detail in Reference [14].

Plasmids: pUC18 and pUC19 from Life Technologies, Inc (www.lifetech.com), pBAD/HisA from Invitrogen, pHK724 from author, and pBAD-GFPuv from Clontech, Inc (www.clontech.com).

DNA polymerase: eLongase from Life Technologies, Inc, Gaithersburg, MD.

Amplification of luxR and gfp genes: The plasmid pHK724 is used for the *luxR* gene template and plasmid pBAD-GFPuv is used for the *gfp* gene template. The amplification program consists of: 95°C, 20 s; 50°C, 20 s; 72°C, 60 s; 30 cycles.

luxR and gfp DNA sequence: *luxR* sequence and *gfp* sequence obtained from the author.

Primer synthesis: Primers synthesized by Life Technologies, Inc.

Growth medium: L-broth is low salt Luria-Bertani medium. LA is L-broth solidified with 1.5% (w/v) Bacto-agar. Their preparation is described in Reference [14].

Antibiotics: Chloramphenicol (Cm) and ampicillin (Ap) from Sigma (www.sigma.aldrich.com). Preparation and concentrations are described in Reference [14].

Arabinose: L-arabinose (ara) from Sigma. Stock concentration is 20% and final concentration is 0.2%. Filter-sterilize.

PCR product purification: The PCR product is purified using the gene cleanup kit from Geno Technology, Inc, St Louis, MO, USA.

Plasmid isolation and purification: Described in Reference [14].

GasPak anaerobic system: GasPak jar and palladium catalyst from Becton Dickinson, Microbiology Systems, Bedford, MA, USA.

Low gelling temperature agarose (LGTA): Low melt preparative grade agarose from Bio Rad Laboratories (www.bio-rad.com).

Recipes for LGTA ligation protocol [13]: $1 \times$ TAE buffer is the working solution of Tris-Acetate-EDTA buffer. $50 \times$ TAE stock buffer is made by dissolving 242 g of Tris base in 700 ml of distilled H_2O. Add 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA (pH 8.0) and bring to final volume of 1 liter with distilled H_2O; ethidium bromide (EtBr) concentration is 10 mg per ml in distilled H_2O; loading dye (LD) is prepared by combining 0.25 g bromophenol blue and/or 0.25 g xylene cyanol, 49 ml distilled H_2O, 1 ml of 1 M Tris (pH 8.0) and 50 ml glycerol; ligation mixture is prepared by adding 5 μl sterile distilled H_2O, 4 μl ligation buffer, and 1 μl T4 DNA ligase (Life Technologies); TCM is prepared by adding 1 ml of 1 M Tris (pH 7.4), 10 ml of 1 M $CaCl_2$, 1 ml of 1 M $MgCl_2$, and 88 ml sterile distilled H_2O.

Low gelling temperature agarose (LGTA) ligation [13], modified by Slock: For 100 ml of LGTA, mix 100 ml $1 \times$ TAE with 0.7 g low melt preparative grade agarose. Melt the gel at 90°C, cool at 37°C for 10 min, then add 5 μl EtBr. Pour the gel and let set for 1 h or longer in cold room. Mix 0.1–2.0 mg of restriction endonuclease-digested DNA plus 1% loading dye in a microfuge tube and load samples to each well. Add double-digested vector DNA to one well, double-digested PCR-amplified DNA to another well and 1-kb ladder DNA to a third well: pUC18 and pUC19 are added to different wells. Run gel in the cold (4°C) at lower than 75 mA. Excise the appropriate DNA bands with a sterile razor blade while viewing the gel under long-wave length UV light. Expose the gel to UV light for as short a time as possible and keep the gel slice as small as possible. Put each gel slice into a different sterile 1.5-ml microfuge tube and liquefy the gel (65–70°C for 5–15 min). Combine an appropriate amount of DNA/LGTA from the vector tube with the insert tube to a final volume of 10 μl. Usually a 1:2 vector to insert ratio is desired. Remelt the chimeric tube (65°C), mix and cool to 37°C for 10 min or longer. Add 10 μl of ice-cold ligation mixture, mix and incubate at room temperature overnight. Remelt the chimeric tube (at 65°C for 10 min) and add 100 μl of ice-cold TCM. Mix 20 μl of chimeric DNA/TCM mixture with 50 μl of competent cells eg, *E. coli* pHK555. Set on ice for 20 min. Heat shock at 42°C for 1 min, cool on ice for 1 min, add 400 μl of L-broth and incubate at 37°C for 1 h. Plate various aliquots on selective media (eg, L-agar/Cm/Ap). Incubate at 28–30°C for 48 h and observe bioluminescent colonies in a darkroom (let your eyes dark adapt for 5 min).

Experiments

Directional cloning of PCR-amplified luxR gene into plasmid vectors

The protocol (and results) for directional cloning of the *luxR* gene into the plasmid vectors pUC18 and pUC19 are shown in Figure 2. Directional cloning requires the selection of two restriction endonuclease (RE) sites from the multiple cloning site of the vector [13]. The plasmid vectors pUC18 and pUC19 were chosen because each has the same multiple cloning site but in the opposite orientation. The two ends of the *luxR* gene (the insert) must contain the same RE sites. The RE recognition sequences are added to the ends of the primers that are used to amplify the *luxR* gene (Figure 3). The vector and insert (*luxR*) gene are digested with both REs in a 'double' digest. Following ligation, the *luxR* gene will be cloned in the proper orientation in one of the vectors but not the other. The RE sites selected must not occur in the insert (*luxR*) sequence. Another consideration is that the insert DNA must be 'in frame' with the vector sequence (Figure 4), so that the codon reading frame is maintained. To visualize this, students cut a long strip of adding machine roll paper and secure it to their work bench. They then write the multiple cloning site sequence (both top and bottom strands) on the paper strip. They must begin with the start codon, ATG, followed by the remaining triplet codons. Next they delineate the RE recognition sequence and with a scissors cut out the sequence at the point of cleavage between the two RE sites. The same is done to the ends of the insert gene and the insert is fitted into the complementary overhangs of the vector. The sequence is then checked for in-frame alignment. REs that produce staggered ends are chosen so that 'sticky' overhangs are generated (Figure 4). The advantage of directional cloning (using two different REs) is that only the vector and insert can ligate. There is no self-ligation of the vector, provided the small fragment of the multiple cloning site generated by the double digest is removed. This is accomplished by Low Gelling Temperature Agarose (LGTA) ligation. LGTA ligation is used to separate the desired products of the double digest (ie the sticky end vector and insert) from the unwanted products.

At the completion of this experiment, students will have learned the following: set up a PCR, design complementary primers to target DNA, design primers with RE recognition sites that will be in frame with vector DNA sequence, use REs for cloning, use LGTA to purify products of an RE digest, use LGTA/DNA to ligate insert with vector to construct a chimeric plasmid, and transform a chimeric plasmid into an *E. coli* host.

Comparison of Lux and GFP systems

Lux bioluminescence and GFP fluorescence when expressed in *E. coli* are compared in Table 1. Lux is the

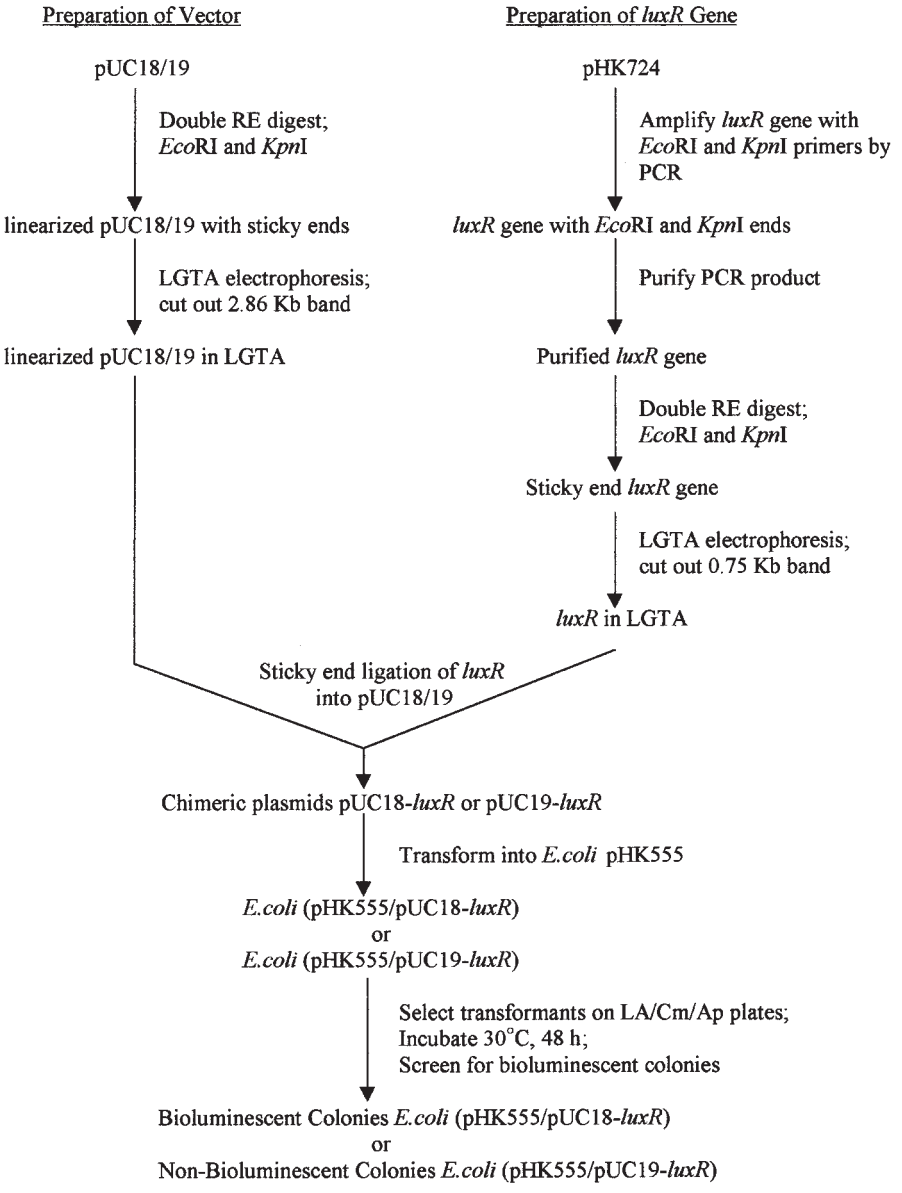

Figure 2 Protocol for directional cloning of *luxR* gene into pUC vectors.

more complicated of the two systems because it requires two plasmids and a total of seven genes. One plasmid, a chimeric molecule, is constructed by amplifying the *luxR* gene and ligating the PCR product into a plasmid vector (eg, pUC18-*luxR*, pBAD/HisA-*luxR*). The other plasmid, pHK555, contains the structural genes for light production. When a pUC18-*luxR* or pBAD/HisA-*luxR* is transformed into *E. coli* pHK555, the resultant colonies (transformants) produce light. The complexity of Lux creates a great teaching opportunity. To involve students in the learning process, they can complete a diagram of *E. coli* (pHK555/pHK724) to show the molecular and cellular events that are necessary to produce bioluminescence [14]. Information regarding transcription, translation, *lac* operon, and *trp* operon would be provided in the lecture portion of the course.

Construction of LuxR-GFP fusion protein

Fusion proteins containing GFP are powerful tools in cell biology which serve as molecular reporters [4]. In the last few years hundreds of publications have documented the construction of such fusions.

An ambitious project using *Caenorhabditis elegans* involves making GFP-fusion proteins of all the roundworm's 19 099 genes. The construction of a LuxR-GFP fusion protein provides the skills and knowledge to learn this important technique. The protocol for making this fusion is similar to that shown in Figure 2 except the protocol is repeated twice. The *luxR* gene is amplified by PCR with primers containing *Nco*I RE sites (Figure 5). The amplified product is purified, digested with *Nco*I and subjected to LGTA electrophoresis. The plasmid vector for this experiment is Invitrogen's expression vector pBAD/HisA

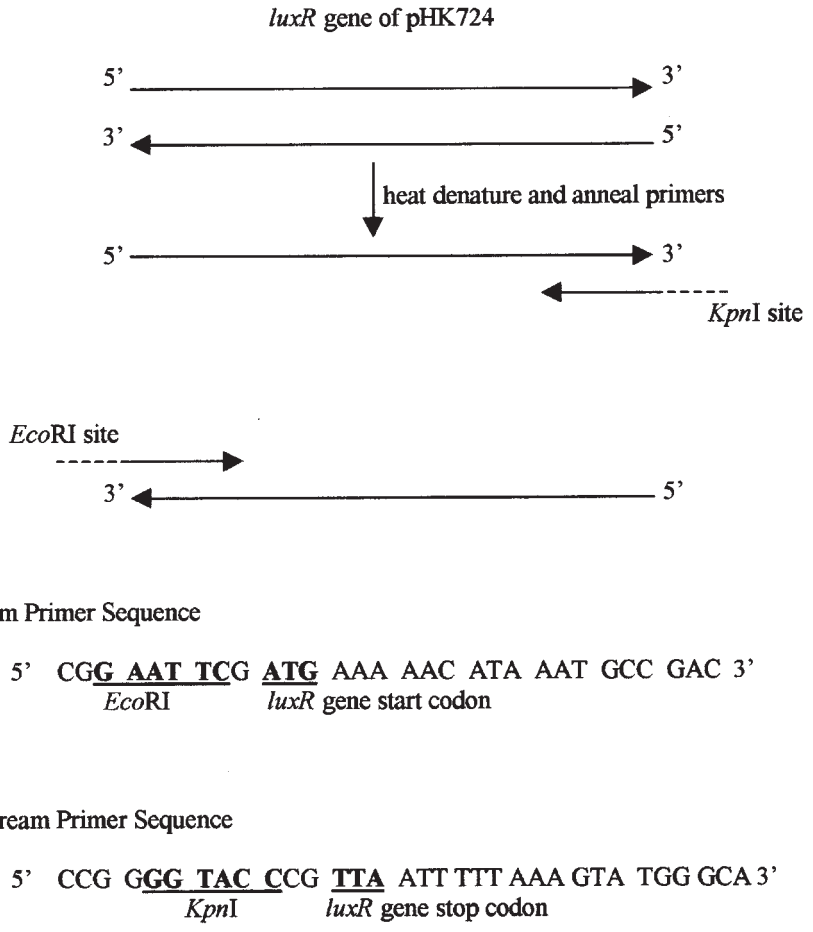

Figure 3 PCR amplification of *luxR* gene and *luxR* gene primer sequences for insertion into pUC18 and pUC19.

(Figure 6). The vector is digested with *Nco*I and subjected to LGTA electrophoresis. The LGTA/DNA bands (4.1-kb vector and 0.75-kb *luxR*) are ligated, the chimeric plasmid transformed into *E. coli* pHK555 and plated on selective media (LA/Cm/Ap/ara). The selective media must contain arabinose (ara) because expression is under the control of an araBAD promoter (Figure 6). Since this is a nondirectional cloning event, a bioluminescent colony is picked and the chimeric plasmid (pBAD/HisA-*luxR*) is isolated by a plasmid isolation procedure.

The protocol is repeated for the *gfp* gene. The *gfp* gene is amplified by PCR with primers containing *Bgl*II and *Eco*RI RE sites (Figure 5). The amplified product is purified and both *gfp* gene and vector pBAD/HisA-*luxR* are double digested with *Bgl*II and *Eco*RI REs. The LGTA protocol is used to ligate the *gfp* insert (0.714 kb) to the vector pBAD/HisA-*luxR* (4.85 kb). The chimeric plasmid (pBAD/HisA-*luxR-gfp*) is transformed into *E. coli* pHK555 and plated on selective media (LA/Cm/Ap/ara). Since the *gfp* gene is cloned directionally into the *Bgl*II/*Eco*RI RE sites of pBAD/HisA-*luxR*, all colonies show the double phenotype of *lux* bioluminescence and *gfp* fluorescence. This LuxR-GFP protein fusion creates a forty-amino acid 'linker' between the C-terminus of the LuxR protein and the N-terminus of GFP. LuxR-GFP fusion proteins with short amino acid linkers do not fluoresce.

Directional cloning of gfp gene

The directional cloning of the *gfp* gene into pBAD/HisA is the same as described in the LuxR-GFP fusion protein section. The primer pair for *gfp* amplification is shown in Figure 5. The amplified product is purified and both *gfp* gene and vector pBAD/HisA (Figure 6) are double digested with *Bgl*II and *Eco*RI REs. The chimeric plasmid (pBAD/HisA-*gfp*) is transformed into *E. coli* and plated on selective media (LA/Ap/ara). Since the *gfp* gene is cloned directionally into the *Bgl*II/*Eco*RI sites of pBAD/HisA, all colonies show GFP fluorescence. This construct (pBAD/HisA-*gfp*) results in a fusion protein (polyhistidine-GFP) that permits GFP expression and purification by affinity chromatography. The N-terminal polyhistidine tag of pBAD/HisA (Figure 6) forms a metal-binding site for affinity purification of the GFP-fusion protein on a metal-chelating resin (pBAD/HisA plasmids). The enterokinase cleavage site (Figure 6) allows for removal of the N-terminal peptide by enterokinase and subsequent release of GFP.

Summary and undergraduate curriculum

We have described a series of advanced PCR techniques using *lux* and *gfp* genes. Directional cloning/expression of a gene and construction of fusion proteins reflects what

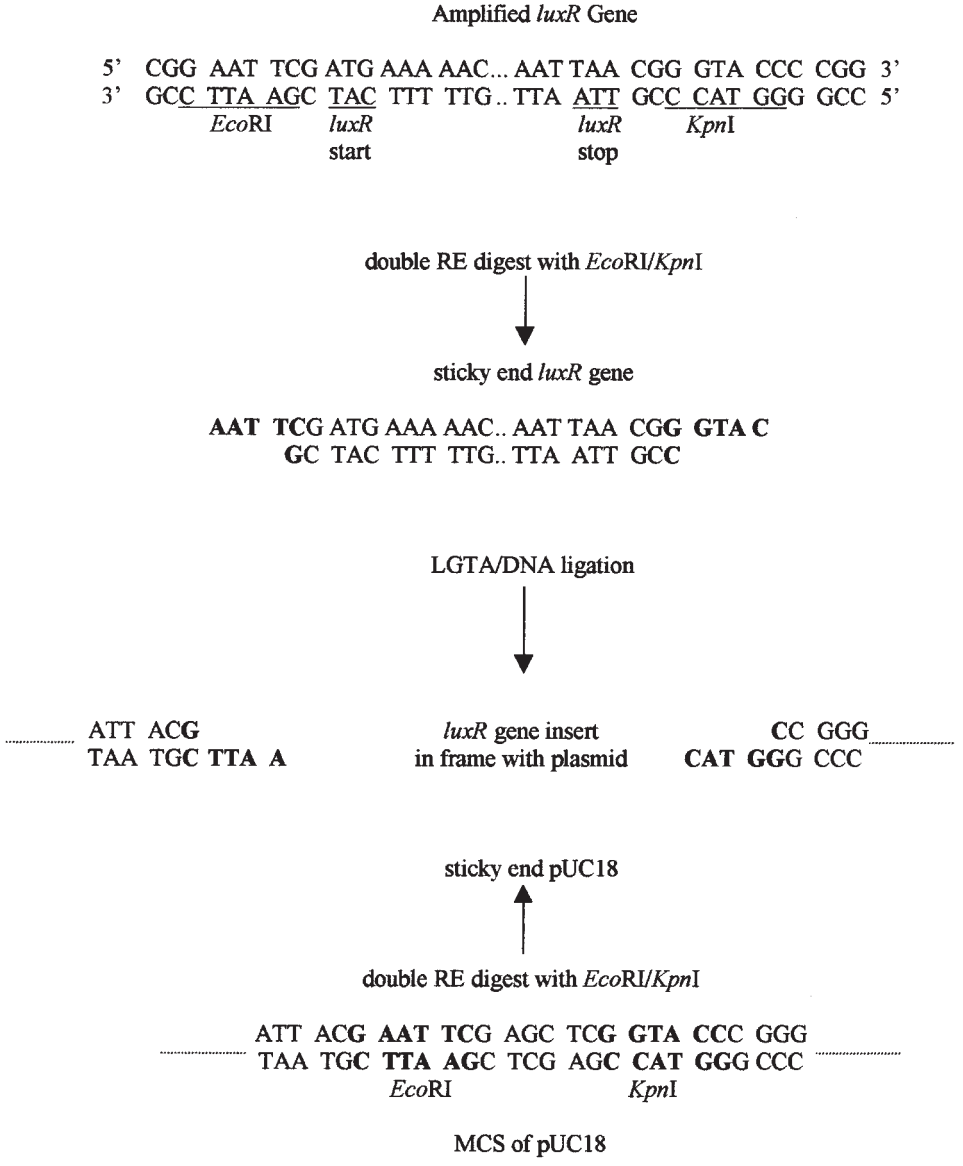

Figure 4 Sticky end ligation of *luxR* gene into pUC18. Bold-face letters indicate RE sites.

Table 1 Comparison of Lux bioluminescence and GFP fluorescence when expressed in *E. coli*

Characteristic	Lux	GFP
1. Color of light	bluish-white	green but variants are blue, yellow and cyan
2. External requirements for light emission	none; except must view in dark room with dark-adapted eyes	requires excitation by UV light
3. Intrinisic requirements for light emission	six additional genes plus *luxR*	none
4. Duration/stability of light emission	lasts 5–9 days; stops after nutrient depletion	lasts months as long as plates do not dry out
5. Temperature dependency for light emission	narrow 18–30°C	wide 4–70°C
6. O_2 dependency for light emission	requires O_2	none; some O_2 needed for chromophore formation
7. Size of expressed protein	LuxR = 250 amino acids	GFP = 238 amino acids

luxR gene primers for insertion into *Nco*I site of pBAD/HisA

Upstream Primer Sequence

5' CAT G**CC ATG G**CG **ATG** AAA AAC ATA AAT GCC GAC 3'
*Nco*I *luxR* start codon

Downstream Primer Sequence

5' CAT G**CC ATG G**CA TTT TTA AAG TAT GGG CAA TCA 3'
*Nco*I ↑ no *luxR* stop codon

gfp primers for insertion into MCS of pBAD/HisA-*luxR* and pBAD/HisA

Upstream Primer Sequence

5' GA**A GAT CT**C C**AT G**GC TAG CAA AGG AGA AGA A 3'
*Bgl*II *gfp* start codon

Downstream Primer Sequence

5' CG**G AAT TC**C **TTA** TTT GTA GAG CTC ATC CAT 3'
*Eco*RI *gfp* stop codon

Figure 5 Primer sequences of *luxR* gene and *gfp* gene show corresponding RE sites for in-frame insertion into pBAD/HisA. Note: Downstream primer of *luxR* gene does not have stop codon so synthesis of LuxR-GFP fusion protein termination occurs at C-terminus of GFP.

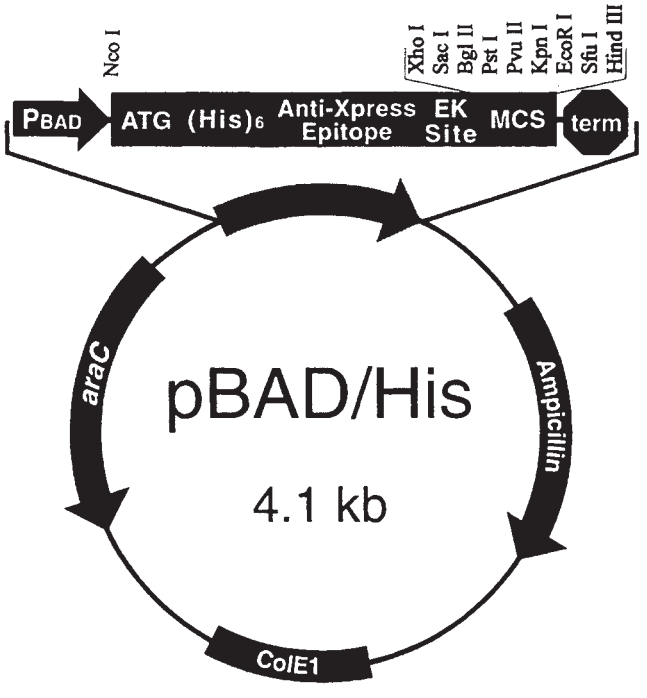

Figure 6 Plasmid pBAD/HisA showing *Nco*I RE site for insertion of *luxR* gene and *Bgl*II/*Eco*RI RE sites of MCS for insertion of *gfp* gene.

molecular biologists actually do in their laboratories. The important difference in these experiments is that along with being instructive they are also visually appealing. A typical reaction from our students upon seeing bioluminescent colonies is to wave their plates in the air like 4th of July sparklers.

The result of our passion for *lux* and *gfp* is the liberal use of these genes in undergraduate laboratory courses. Students in introductory biology perform a bacterial transformation using *luxR* [14] and *gfp* plasmids. As stated in the LGTA ligation protocol (and Table 1) most plates are incubated at 30°C. A few plates of *lux* and *gfp* are also incubated at 37°C. Students can evaluate the influence of temperature on microbial growth. *E. coli* will grow at both temperatures but faster at 37°C. However, the *lux* genes are from the marine bacterium, *V. fischeri*. *V. fischeri* has adapted to a cooler environment and, therefore, the proteins for bioluminescence are heat-denatured at 37°C. GFP expression is unaffected by these temperature changes. The O_2 requirement (Table 1) for bioluminescence and fluorescence can also be studied. Two-day-old plates (30°C) are placed in an anaerobic jar (Gas Pak system). Within a few hours the *lux* bioluminescence is extinguished, but the GFP is unaffected. Upon opening the jar and exposing the *lux* plates to air (O_2), bioluminescence returns to full strength within 5 min ($FMNH_2 + O_2 + RCHO \rightarrow FMN + RCOOH + H_2O + LIGHT$).

Sophomore biochemistry students perform a series of seven experiments designed to isolate, purify and characterize GFP from a crude protein extract [16]. Working in small groups, students purify GFP nearly a thousand-fold. They then complete a purification table, characterizing GFP with respect to purity, charge, hydrophobicity and molecular weight. Affinity chromatography purification of recombinant GFP (pBAD/HisA-*gfp*) is also performed. The value of GFP as a teaching tool lies in its ease of detection. One can always find GFP in a fraction tube or a colony on a plate by illumination with a hand-held UV lamp.

The three directional cloning experiments described in this article are the major components of the molecular genetics lab that is taught at the junior/senior level. A fourth component of the lab is a site-directed mutagenesis (SDM) experiment. In this experiment a point mutation in the *luxR* gene of pHK724 is mutated back to wild-type using SDM. All of these experiments use PCR to provide hands-on experience in cloning, expression and manipulation of genes.

Cultures and plasmid pHK724

The bacterial strain *E. coli* pHK555 and plasmid pHK724 necessary to conduct these experiments may be obtained free of charge by writing to the corresponding author.

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