

Production and Purification of Recombinant Fluorescent Protein

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ABSTRACT: Fluorescent proteins are genetically encoded, easily imaged reporters crucial in biology and biotechnology. The fluorescent proteins from the jelly fish *Aequorea Victoria*, has become a versatile reporter for monitoring gene expression and protein localization in the variety of cells and organisms. The fluorescent proteins (cherry gene) can be introduced into *Escherichia coli* and maintained their genome through injection with a viral vector or cell transformation. Transformants were obtained by the transformation procedure. After transformation, the bacterial transformants were confirmed using agarose gel electrophoresis. Normally the fluorescent proteins are hydrophobic in nature. So the cherry proteins were purified by hydrophobic interaction chromatography. After purification, the amounts of fluorescent proteins were estimated by Bradford method. Finally the maximum absorbance value of fluorescent (cherry) proteins were identified. This would be identified by maximum gene expression of the (cherry) fluorescent protein.

KEYWORDS: Gene expression, protein localization, transformation, hydrophobic interaction chromatography.

I. INTRODUCTION

The discovery of green fluorescent protein in the early 1960s ultimately heralded a new era in cell biology by enabling investigators to apply molecular cloning methods, fusing the fluorophore moiety to a wide variety of protein and enzyme targets, in order to monitor cellular processes in living systems using optical microscopy. The recent technical advances in widefield fluorescence and confocal microscopy, including ultrafast low light level digital cameras and multitracking laser control systems, the green fluorescent protein and its color-shifted genetic derivatives have demonstrated invaluable service in many thousands of live-cell imaging experiments. Osamu Shimomura was the first person to isolate GFP (1960) and to find out which part of GFP was responsible for its fluorescence. He first isolated a calcium dependent bioluminescent protein from the *Aequorea victoria* jelly fish, which they named aequorin, a second protein was observed that lacked the blue exciting bioluminescent properties of aequorin, but was able to produce green fluorescence when illuminated with UV light due to this property, the protein was eventually named GFP. It is important to note that the native green fluorescent protein fluorophore exists in two states. A protonated form, the predominant state, has an excitation maximum at 395 nanometers, and a less prevalent, unprotonated form that absorbs at approximately 475 nanometers. Regardless of the excitation wavelength, however, fluorescence emission has a maximum peak wavelength at 507 nanometers, although the peak is broad and not well defined. Denaturation of GFP requires treatment with 6 M guanidine hydrochloride at 90 °C, or pH outside the range 4-12. Renaturation occurs within minutes following reversal of denaturing conditions by dialysis or neutralization. Green fluorescent protein and the mutated allelic forms, blue, cyan and yellow fluorescent proteins are used to construct fluorescent chimeric proteins that can be expressed in living cells, tissues and entire organisms, after transfection with the engineered vectors. Red fluorescent proteins have been isolated from other species, including coral organisms and are similarly useful. The integration of GFP into *Escherichia coli* cells has been used to measure bacterial response to stress by monitoring the intensity of fluorescence in a fed batch bioreactor. GFP expressing bacterial cells have been

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demonstrated to be no less viable than corresponding wild type cells . In live cells, fluorescent proteins are most commonly employed to track the localization and dynamics of proteins, organelles, and other cellular compartments. A variety of techniques have been developed to construct fluorescent protein fusion products and enhance their expression in mammalian and other systems. The primary vehicles for introducing fluorescent protein chimeric gene sequences into cells are genetically engineered bacterial plasmids and viral vectors. The basic plasmid vector configuration useful in fluorescent protein gene transfer experiments has several requisite components. The plasmid must contain prokaryotic nucleotide sequences coding for a bacterial replication origin for DNA and an antibiotic resistance gene. These elements, often termed shuttle sequences, allow propagation and selection of the plasmid within a bacterial host to generate sufficient quantities of the vector for mammalian transfections. In addition, the plasmid must contain one or more eukaryotic genetic elements that control the initiation of messenger RNA transcription, a mammalian polyadenylation signal, an intron, and a gene for co-selection in mammalian cells. Transcription elements are necessary for the mammalian host to express the gene fusion product of interest, and the selection gene is usually an antibiotic that bestows resistance to cells containing the plasmid. These general features vary according to plasmid design, and many vectors have a wide spectrum of additional components suited for particular applications.

The gene for the production of GFP is spliced into the genome of the organism in the region of the DNA which codes for the target proteins, and which is controlled by the same regulatory sequence; that is the gene's regulatory sequence now controls the production of GFP, in addition to the tagged proteins .In cells where the gene is expressed, and the tagged proteins are produced, GFP is produced at the same time. Thus, only those cells in which the tagged gene is expressed, or the target proteins are produced, will fluoresce when observed under fluorescence microscopy. Analysis of such time lapse movies has redefined the understanding of many biological processes including protein folding, protein transport, and RNA dynamics, which in the past had been studied using fixed material. The powerful use of GFP is to express the protein in small sets of specific cells. This allows researchers to optically detect specific types of cells *in vitro*, or even *in vivo*. Genetically combining several spectral variants of GFP is a useful for the analysis of brain circuitry. The fluorescent Protein techniques avoids the problem of purifying tagging and introducing labeled proteins into cells or the task of producing specifying specific antibodies for surface or internal antigens. Recently Fluorescent Proteins from other species have been denotified and isolated resulting in further expansion of the color palette. With the rapid evolution of Fluorescent protein technology, the utility of this genetically encoded fluorophore for a wide spectrum of applications beyond the simple tracking of tagged biomolecules in living cells.

II. MATERIALS AND METHODS

A systematic study was undertaken to isolate and purify the fluorescent protein, capable of expressing the gene and to estimate the protein for its maximum concentration.

METHODS

1. TRANSFORMATION

Transfer 1ml of bacterial culture into a fresh microfuge tube (two tubes – one for negative control and the other for positive control). Centrifuge at 3000 rpm for 3 minutes. Carefully discard the supernatant. To the pellet add 100 µl of solution I (TSS and DMSO). Mix the pellet gently. Add 2µl of plasmid to the tube. Gently mix with a and store the tube on ice for 30 minutes .After the incubation time, add 900 µl of solution II (TSS). Shake the tube at 225 rpm at 37⁰C for 1 hour. Centrifuge the tubes at 3000 rpm for 4 minutes. Gently remove 900µl of the supernatant and discard it. Resuspend the pellet with the remaining solution in the tube using a pipette. Pipette out 100µl of this solution onto LB agar plate with ampicillin plated on it. Use a clean sterilized spreader to spread the culture uniformly on the plate to obtain single colonies. Incubate the plate overnight in an incubator at 37⁰C.

2. PREPARATION OF PLASMID DNA BY ALKALINE LYSIS WITH SDS

Pour 1:5 ml of the culture into a microfuge tube. Centrifuge at maximum speed for 3 minutes in a microfuge. Pour off the medium, leaving the bacterial pellet as dry as possible. Resuspend the bacterial pellet in 100µl of ice-cold resuspension buffer by vigorous vortexing. Add 200µl of freshly prepared lysis buffer to each bacterial suspension. Close the tube tightly, and mix the contents by inverting the tube rapidly five times. Add 150µl of ice-cold

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neutralization buffer. Close the tube and disperse through the viscous bacterial lysate by inverting the tube several times. Store the tube on ice for 3-5 minutes. Centrifuge the bacterial lysate at maximum speed for 5 minutes in a microfuge. Transfer the supernatant to a fresh tube. Precipitate nucleic acids from the supernatant by adding 2 volumes of 100% ethanol at room temperature. Mix the solution by overtaxing and then allow the mixture to stand for 2 minutes at room temperature. Collect the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge. Remove the supernatant. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Add 1 ml of 70% ethanol to the pellet and invert the closed tube several times. Pellet the DNA by centrifugation at maximum speed for 5 minutes in a microfuge. Remove all of the supernatant. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (5-10 minutes). Resuspend the pellet in 30µl of TE (pH 8.0) containing 20 µg/ml RNase A (pancreatic RNase). Vortex and Store the DNA solution at 20°C.

3.DIGESTION OF PLASMID DNA WITH RESTRICTION ENDONUCLEASES

In a microfuge tube, placed 13µl of deionized water, 2µl of reaction buffer, 4µl of plasmid miniprep, 1.0µl of ECOR1. The enzymes and buffer were stored in a freezer. The tube was tapped to mix the contents well and it was pulsed down in a micro centrifuge. The tube was incubated in an incubator for 1 hour. Finally the tube was stored in the freezer and prepared for agarose gel electrophoresis.

4.AGAROSE GEL ELECTROPHORESIS FOR VISUALIZING PLASMID DNA

1% agarose gel in Tris-acetate EDTA buffer (1x TAE) containing ethidium bromide (EtBr) was prepared. To 1gm of agarose, add 100ml of 1x TAE and heated until dissolved then Cool the gel to 50°C and add ethidium bromide (0.5mg/ml) before pouring into the gel apparatus. Place the apparatus on a level surface and check with the spirit level and adjust the level. Appropriate comb was placed. Gel was poured and allowed for solidification. After gel has set firmly, poured little amount of buffer and removed the comb gently. Immersed the gel slowly into the gel tank. Added sufficient amount of 1x TAE buffer and the electrodes were connected. The digested plasmids was loaded into the wells. sufficient amount of molecular weight markers was added. The gel was run at 100 volts.

5.FLUORESENT PROTEIN PURIFICATION

Set up primary cultures of bacteria carrying the gene for the green fluorescent protein. Take 2 ml of LB media and add ampicillin (100mg/ml) to get a final concentration of 50µg/ml. Inoculate media with the bacteria. Incubate overnight in a shaker at 37°C. Setup up a secondary culture and induce with Arabinose (6mg/ml) after two and a half hours. Let the culture grow over night and process the next day. Pellet the culture down at max speed for 3min. Add 250µl of Tris EDTA buffer to the pellet and resuspend the pellet by rapidly pipetting up and down several times. Add one drop of lysozyme to the resuspended solution. Mix the contents gently by flicking the tube. Place the tube in ice for half an hour. Pellet the mixture at max speed for 10min. Prepare the chromatography column Remove the cap and let the buffer in the tube drain out Add 2*1 ml of equilibration buffer to the column Drain the buffer to the 1ml mark on the column and cap the column. Label three collection tubes (1,2,3). Transfer 250 µl of the lysate to a fresh tube, and add 250 µl of Binding buffer to it. Carefully and gently load 2*250 µl of the lysate mixed with binding buffer. Remove the caps from the top and bottom of the column and place the column in collection tube 1. After it stops dripping transfer the column to collection tube 2. Add 250 µl of wash buffer and let the entire volume flow into the column. After the column stops dripping, transfer it to tube 3. Add 750 µl of TE Buffer and let the entire volume flow into the column. Examine all three collection tubes and note any difference in color between the tubes store the tubes in 20°C.

6.ESTIMATION OF PROTEIN USING BRADFOED REAGENT

Gently mix the bradfoed Reagent solution and equilibrate to room temperature. Pipette 40µl of each sample to be assayed into appropriately labeled test tubes. Add 2ml of bradfoed reagent to each tube and mix well. Incubate the tubes at room temperature for 5min. Transfer the sample into the cuvette. Set the spectrophotometer at 595 nm. Measure the absorbance of the standards and sample solutions. Create a standard curve by plotting the absorbance at 595nm vs. protein concentration of each protein standard (mg/ml). Determine the protein concentration of unknown samples by comparing their absorbance values against the standard curve.

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III.RESULTS

TRANSFORMATION OF BACTERIAL CELLS

The bacterial transformation is the process of intercellular transfer of genetic information in which a small portion of the total DNA of a lysed bacterium enters a related bacterium and is incorporated into the DNA genome of the recipient. In this transformation method, transformed colonies were obtained. The fluorescent protein (cherry) gene was introduced into *Escherichia coli* and maintained their genome through injection with a viral vector or cell transformation. In this step, the *Escherichia coli* strains DH5 α are sensitive to antibiotics such as ampicillin, Cherry plasmid genes are resistant to ampicillin. Thus if the bacterial transformation is plated into media containing ampicillin, only bacteria which possess the cherry plasmid will have the ability to metabolize ampicillin and form transformed colonies. In this step, other non transformants also present in LB agar plate. Satellite colonies are non transformed Colonies. But showing this colonies are similar to transformed colonies. So that transformed colonies were selected by antibiotic selection method.

PLASMID PREPARATION BY USING AGAROSE GEL ELECTROPHORESIS

During transformation, the gene expression were identified. The cherry gene encodes for the cherry Protein. This gene expression were succeeded by transformation method. But it should confirmed the bacterial transformants by using agarose gel electrophoresis. In this method, linearised, circular, supercoiled bands will form. linearised band were identified by using restriction digestion method. The cherry plasmid DNA were digested by single digestion method. The linearised band were confirmed the bacterial transformants.

HYDROPHOBIC INTERACTION CHROMATOGRAPHY

The purification of fluorescent protein by using hydrophobic interaction chromatography. To isolate the cherry protein and to purified the fluorescent protein by using hydrophobic interaction column. 3 different concentration of buffers are used. All buffers have 10M ammonium sulphate. Normally the fluorescent proteins are more hydrophobic in nature. Hydrophobic residues are present inside the cell. This high concentration of ammonium sulphate buffers are react with the equilibration buffer (2M) to expose the hydrophobic residues from the cell. Equilibration buffers to equilibrate the column. Binding buffer (4M) bind to the lysate. This buffer helps to the protein bind to the column some of the other bacterial proteins have also hydrophobic in nature. But fluorescent proteins are more hydrophobic in nature. So the fluorescent proteins are more interact to the column. Other proteins are washed away from the column by using washing buffer (1.3M). Finally elution buffer were added, the purified fluorescent protein will be eluted from the column the protein of interest (cherry fluorescent protein) were obtained.

FLUORESCENT PROTEIN ESTIMATION BY USING BRADFOED REAGENT

TABLE 1

S.No.	Concentration of BSA (ug)	OD Value at 595nm
1	200	0.179
2	400	0.303
3	600	0.452
4	800	0.513
5	1000	0.623

TABLE 2

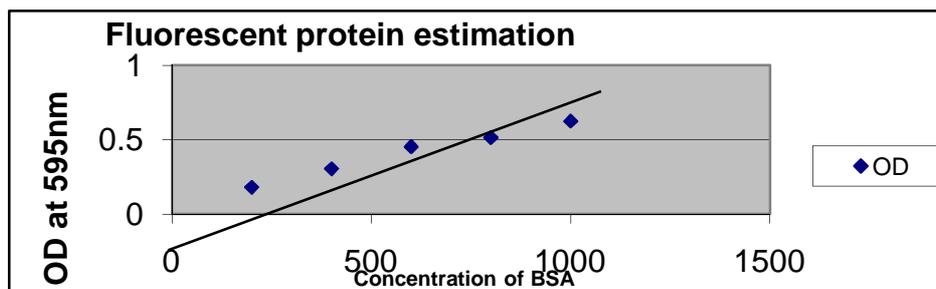
S.No.	Sample	OD Value at 595nm
1	Direct Sample	0.031
2	Indirect Sample 1:1	0.111
3	Indirect Sample 1:10	0.177

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FLUORESENT PROTEIN ESTIMATION BY BRADFOED METHOD



This graph showing that to obtain the maximum amount of protein for its maximum concentration. Bovine serum albumin (BSA) Stock standard (1mg/mL) were prepared and make to 0.2, 0.4, 0.6, 0.8 1mg/mL. add 2 ml brad foed's reagent. To read the OD value at 595 nm. The direct sample (protein of interest (40ul) and indirect sample 1 : 1 dilution (250ul distilled water and 250ul sample), 1:10 dilution, (50ul of sample and 450ul of distilled water,) were prepared for that indirect sample and direct sample to make each 40ul and to add 2 ml of bradfoeds reagent. Finally to read the OD value at 595 nm. This graph value showing that the maximum amount of protein for its maximum concentration.

CALCULATION:

40ul of (1:10 dilution) indirect sample contains 40ug of BSA.

1000ml of indirect sample contains = $4000 \times 40 / 40$

(1:10 dilution) = 1000×10

= 10,000 mg/ml of cherry

fluorescent protein.

40ul of (1:1 dilution) indirect sample contains 144 ug of BSA.

1000ml of indirect sample contains = $1000 \times 144 / 40$

= 3600×1

= 3600 mg/ml of cherry

fluorescent protein.

40 ul of direct sample contains 240 ug of BSA.

1000ml of direct sample contains = $1000 \times 240 / 40$

= 6000 ug/ml

1 ml contains

= $6000 / 10000$

= 6mg / ml of cherry fluorescent

protein.

EXCITATION PEAK VALUE OF THE FLUORESCENT PROTEIN

TABLE 3

Absorbance	Optical density Value
400	0.054
450	0.037
500	0.067
550	0.192
600	0.200
650	0.008

The BSA standard (0.2, 0.4, 0.6, 0.8 1mg/ml, direct sample, 1:1, 1:10, (indirect sample) for that to add 2ml of bradfoeds reagent to set the OD value at 400, 450, 500, 550, 600, 650. Finally to identify that maximum absorbance value of the fluorescent protein.550 absorbance value will be maximum.

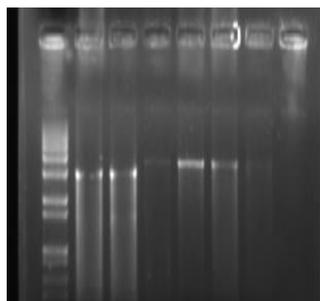
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(a) Ampicillin coated LB plates showing the growth of transformed bacterial cells as single cherry coloured colonies.



(b) Agarose gel showing the presence of Cherry Plasmid in lane 5. The plasmid DNA was mixed with loading dye and electrophoresed through a 1% agarose gel in Trisacetate-EDTA buffer and ethidium bromide. The gel was visualized under UV light. Lane 1 is DNA size standards.



(c) Fig showing the purification of cherry fluorescent protein by Hydrophobic interaction chromatography by using various buffers namely Equilibration buffer (1), Binding buffer (2), Wash buffer (3) and Elution buffer (4).



(d) Fig showing the purified cherry protein fluorescing under UV light.

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IV. CONCLUSION

Organisms from all kingdoms have been transformed with the *Aequorea Victoria* green fluorescent protein (GFP) and biotechnology has been advanced by the use of fluorescent protein. Fluorescent proteins are quite versatile and have been successfully employed in almost every biological discipline from microbiology to systems physiology. These probes have been extremely useful as reporters for gene expression studies in cultured cells and tissues, as well as living animals. In live cells, fluorescent proteins are most commonly employed to track the localization and dynamics of proteins, organelles, and other cellular compartments. Therefore the study was undertaken to isolate and purify the fluorescent protein.

- Isolation and purification of fluorescent protein.
- To find the maximum amount of protein.
- Identify the excitation value of the protein.

It is used to monitoring the gene expression, and make the use of cell biology and molecular engineering.

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