Short-term Research Project, **2018-19**

Title: Designing and construction of a Mammalian Expression Cassette Containing Monomeric Green Fluorescence Protein Gene in pCDNA3.1 by Molecular Cloning Method

By

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DECLARATION

I do hereby declare that the project entitled **"Designing and construction of a Mammalian Expression Cassette Containing Monomeric Green Fluorescence Protein Gene in pCDNA3.1 by Molecular Cloning Method"** is submitted as a summer project for the year 2018-19 has been carried out by me at School of Biological Science (SBS), Indian Association for the Cultivation of Science, under the guidance of Dr. Prosenjit Sen.

Any further extension, continuation or use of this project has to be undertaken with prior express written consent from the Supervisor. I further declare that the project work or any part thereof has not been previously submitted for any degree or diploma in any university.

Name: kripanjali Ghosh
Date:

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My acknowledgement would be incomplete without thanking the biggest source of my strength, my parents for the constant support given to me all the times.

Date: …….

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Designing and Construction of a Mammalian Expression Cassette Containing Monomeric Green Fluorescence Protein in pCDNA3.1 by Molecular Cloning Method

Abstract

Green fluorescence protein (GFP) is a small but a very stable protein which absorbs blue light and fluoresces green. It has a very negligible toxicity to the cell it expresses. Using these characteristics, protein tagging to the N or C terminus of GFP has revolutionized the research field. It has a wide range of application and various advantages in cell biological and proteomics study. Here in this dissertation we have successfully cloned the GFP gene within mammalian constitutive expression vector pcDNA3.1 which can be used to tag any protein of interest to the N or C terminus of GFP to visualize its localization and its interaction with other proteins through confocal microscope or any other fluorescence microscope.

Introduction

Green Fluorescent Protein was discovered by Shimomura et al as a companion protein to aequorin, the famous chemiluminescent protein from *Aequorea* jellyfish [1]. In the 1960s Japanese researcher Osamu Shimomura showed that the *Aequorea victoria* jellyfish that contains a bioluminescent protein called Aequorin. This protein binds with calcium to emit blue light and the blue light is then absorbed by Green Fluorescent Protein. The GFP converts the blue light to green light, making the jellyfish glow green [2]. Its ability as a tool for molecular biology research was recognized in 1992, when Douglas Prasher discovered the GFP gene, & cloned wtGFP and reported its nucleotide sequence. The availability of GFP and its derivatives have thoroughly redefined fluorescence microscopy and the way it is used in cell biology and other biological disciplines. While most small fluorescent molecules such as FITC (Flourescein Isothiocyanate) are strongly phototoxic when used in live cells, GFP is usually much less harmful when illuminated in living cells which widens its applications. GFP can be expressed in different structures, thus enabling morphological distinction [3].

Properties of GFP

- The green fluorescent protein (GFP) is a protein composed of 238 amino acid residues (26.9 kDa) that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range.[4]
- GFP traditionally refers to the protein first isolated from the jellyfish *Aequorea victoria*. The GFP from *A. victoria* has a major excitation peak at a wavelength of 395 nm and a minor one at 475 nm. Its emission peak is at 508-509 nm, which is in the lower green portion of the visible spectrum. The fluorescence quantum yield(QY) of GFP is 0.79
- GFP has a beta barrel structure consisting of eleven β-strands with a pleated sheet arrangement, with an alpha helix containing the covalently bonded chromophore 4-(*p*hydroxybenzylidene) imidazolidin-5-one (HBI) running through the center. [5]
- Five shorter alpha helices form caps on the ends of the structure. The beta barrel structure is a nearly perfect cylinder, 42Å long and 24Å in diameter (some studies have reported a diameter of 30Å), creating what is referred to as a "β-can" formation, which is unique to the GFP-like family [5,6,7]

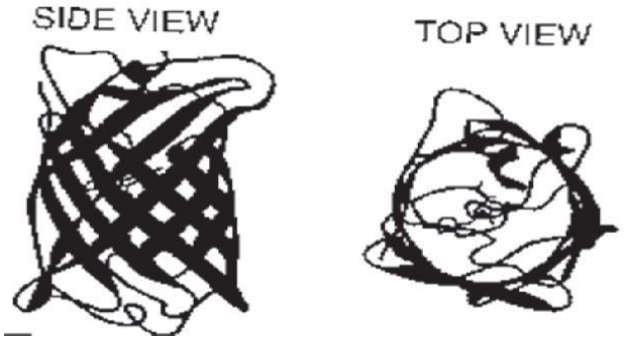


Fig.1: Three-dimensional structure of GFP consisting of 11- stranded -barrel with central -helix
[Adapted from Deo & Daunert, 2001; *Fresenious J Anal Chem* 369: 258-66 (8)].

Applications of GFP

Nowadays, GFP and its variants and homologs of different colours are used in a variety of applications to study the organization and function of living systems (Figure 2). GFP has provided scientists with a rich palette of variants with different biochemical and spectral characteristics, which represent a huge source of potentially powerful molecular tools for numerous applications in the study of complex biological systems. In this section, the basic applications of GFPs are briefly summarized and described. Each of the briefly mentioned applications, however, deserves a separate comprehension to summarize all the information relevant for rational experimental planning.[9] GFP makes for an excellent tool in many forms of biology due to its ability to form internal chromophore without requiring any accessory cofactors, gene products, or enzymes / substrates other than molecular oxygen. [10]

GFP as Reporter Gene:

The GFP *gene is* typically used in the field of cellular and molecular biology as one of the many reporter genes. Basically, a reporter gene is a type of gene that researchers, especially in laboratory experiments, use to attach to a pre-specified sequence of the gene (oftentimes an experimental one) such as that of bacteria, plants, animals, and cell cultures. Some of the criteria that researchers use when selecting a reporter gene include, but may not be limited to, having easily identifiable and selectable markers and their ability to introduce changes that tend to be easily spotted under certain conditions. When conducting laboratory experiments in molecular biology, it would be important for researchers to know the different variables involved and the impact that they create on the experimental environment. Therefore, it makes sense to select reporter genes that possess these qualities such as the GFP. [3]

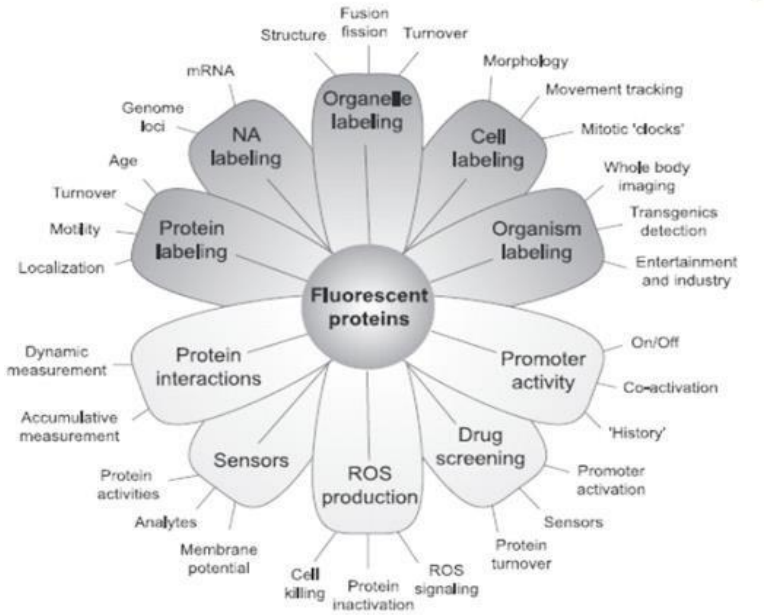


Fig. 2: Main area of application of GFP in Biological science (adapted from Chudakov et al., 2010, [11]).

There are several advantages of using GFP as a reporter over other fluorescent proteins:

1. The formation of fluorophore is a post- translational modification and is either autocatalytic or require some common cell components. No special co-factors or enzymes are required. Thus, theoretically it can be functional in any cell.
2. The fluorophore of GFP is well protected by the secondary structure of the protein and is very stable in many denaturing conditions such as high pH, high temperature, proteases and detergents.
3. The fluorescence of GFP does not require any substrate or other molecules. It can emit green fluorescent light by absorbing incident light or accepting energy from an excited luciferinor photoprotein.
4. GFP can tolerate N- and C- terminal fusion to a broad variety of proteins. Many of proteins have been shown to retain their native function, probably due to the relatively small molecular weight of GFP which reduces its influence on the function of target proteins.
5. GFP is not toxic to transformed cells since it has been expressed in a wide variety of organisms, and cells transformed with GFP vector can grow well.

With these advantages, GFP has wide applications as a reporter for gene expression, protein localization, cell transformation, protein-protein interaction, enzyme activity etc. in bacterial, plant cells, and mammalian cells [7].

GFP as a marker for protein location in fusion proteins:

The most thriving application of GFP is considering it as a genetically fusion partner to host proteins to monitor their localisation and destiny. The gene encoding a GFP is fused in frame with the gene encoding the endogenous protein of interest and the resulting chimera expressed in the cell or organism of interest. GFP can be fused to a host protein to create a fusion protein that usually retains both the fluorescence of the GFP and the biochemical function of the original host. GFP has been targeted successfully to practically every major organelle of the cell, including plasma membrane (27, 23-26), nucleus (28,26,16), endoplasmic reticulum (30, 31, 16), Golgi apparatus (16), secretary vesicles (29, 17), mitochondria (28, 89, 18,19), peroxisomes (20), vacuoles (21), and phagosomes (22). Thus the size and shape of GFP and the differing pH and redox potentials of such organelles do not seem to impose any serious barrier. [5, 12]. In general, fusions can be attempted at either the amino or carboxyl terminus of the host protein, sometimes with intervening spacer peptides.The fused protein maintains its normal function along with acquired fluorescent property through GFP expressing gene.[5] GFP protein can be mutagenized by altering the coding sequence, and the effects of such alterations on the localization or function can be studied in a variety of ways.

As a reporter for DNA and protein localisation, GFP has offered highly sensitive and innovative approaches to study bacterial cell organisation. GFP possesses several characteristics useful for localized bacterial studies. The GFP gene expression is utilised to examine the primary cellular functions such as DNA replication, protein translation and signal transduction [6].

GFP as a reporter for gene expression:

GFP can be co-expressed with a target protein as a reporter for gene expression by inserting GFP gene under the control of the same promoter of the target gene.GFP can also be used to estimate the relative or absolute expression level of the target gene if the relationship between GFP fluorescence and its expression level has been established.[7]

Fluorescence microscopy is one of the most powerful tools for elucidating the cellular functions of proteins and other molecules. When the gene for the production of GFP is incorporated into the genome of the organism in the region of the DNA that codes for the target proteins and that is controlled by the same regulatory sequence; that means, the regulatory sequence of the gene 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 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 etc.

GFP in FRET-based energy transfer:

To study protein-protein interactions and conformational changes of proteins in vivo by Forster or Fluorescence Resonance Energy Transfer (FRET), GFP has been employed as an intracellular molecular sensor [12]. FRET requires at least two kinds of fluorescent proteins with different excitation and emission spectrum. FRET is a process by which excited-state energy is transferred directly from one fluorophore (the ‘‘donor’’) to other nearby molecules (the ‘‘acceptors’’) through near-field electromagnetic dipole interactions [13]. Förster or fluorescence resonance energy transfer (FRET), first described by Theodor Förster in 1946, is a physical phenomenon in which a donor fluorophore in its excited state non-radiatively transfers its excitation energy to a neighboring acceptor fluorophore, thereby causing the acceptor to emit its characteristic fluorescence.[15]

Since FRET is highly sensitive to the distance between donor and acceptor dipoles within the 1–10 nm range, FRET-based biosensors, composed of fluorophores and sensing domains, have been widely adopted as spectroscopic rulers to monitor a variety of biochemical activities that produce changes in molecular proximity, such as protein–protein interactions, conformational changes, intracellular ion concentrations, and enzyme activities [14]. The most general way to make biochemically sensitive GFPs is to exploit fluorescence resonance energy transfer (FRET) between GFPs of different colour. [5]

Mammalian expression vector pcDNA3.1:

pcDNA3.1 is a mammalian expression vector of 5.4kb which are specially designed for high level stable and transient expression of protein in mammalian hosts. pcDNA3.1 is available with the multiple cloning sites in the forward (+) and reverse (-) orientations to facilitate cloning of gene of interest (open reading frame of gene) in which we insert the gene ORF by various enzyme (here *HindIII/NotI*). Many *E. coli* strains are suitable for the growth of this vector easily. The Ampicillin resistance genes in the plasmid pcDNA3.1 allow selection of the plasmid in *E.coli* in the presence of the antibiotic Ampicillin.

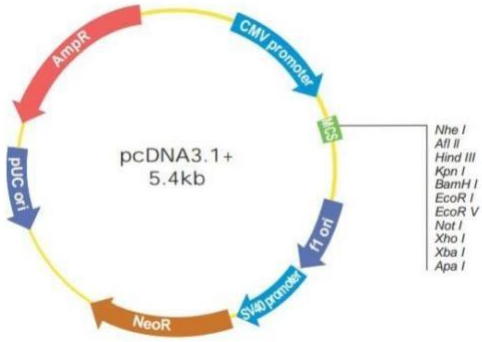
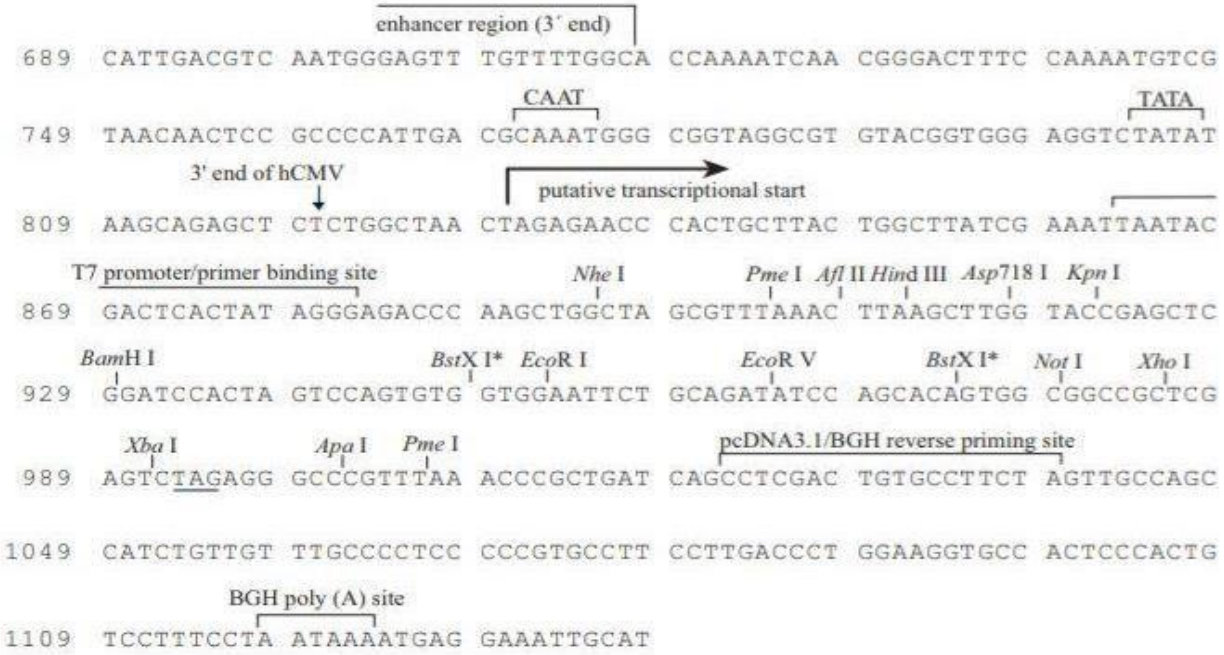


Figure 1: Restriction Map and Multiple Cloning Sites of pcDNA3.1 (5.4 kb) Vector

The Sequence of PCDNA3.1



Here we aimed to construct an expression vector cassette with GFP as reporter gene in pcDNA 3.1 backbone such that any gene can be cloned either upstream (to be cloned with *NheI* and *AflII*) or downstream (to be cloned with *XhoI, XbaI* and *ApaI*) of GFP in order to exploit it in future. We have used mEGFP while doing this work because in some cases EGFP or turboGFP are known to dimerise in particular conditions. Since the prime target of this construction is to study the interactions of proteins or colocalisation of different proteins the homodimer formation between GFP proteins have to be avoid. The user of this construct also would be careful about the brightness and the stableness of FPs [35]. mEGFP has been obtained by a point mutation: critically, the replacement of the hydrophobic residues in the dimer interface of the Aequorea FPs with positively charged residues eliminates the dimer formation without changing the spectral characteristics and localization of protein. The most effective mutation to disrupt the dimer interface in the Aequorea proteins is the A206K substitution, where the hydrophilic lysine residue replaces the non-polar amino acid alanine [13]. Monomeric form have a weak tendency to form dimer. Considering all this factors mEGFP was the best choice.

Objective

Cloning of Monomeric Green Fluorescence Protein (GFP) in mammalian constitutive expression vector pcDNA3.1

Materials and Methods

Vector

mEGFP vector containing GFP gene, and pCDNA3.1(5.4kb) vector were supplied from laboratory (Biological Chemistry Laboratory, School of Biological Sciences, IACS, Kolkata).

Chemicals & Reagents

- All the PCR reagents like taq DNA polymerase, PCR buffer, GFP specific forward and reverse primers, 25mM $MgCl_2$; restriction enzymes *HindIII* and *NotI*, Tango buffer were procured either from Thermo Ficher or from Promega.
- Molecular Ladder used during agarose gel electrophoresis was lamda HindIII DNA ladder Promega. • Reagents used in the preparation of competent cells were procured from Sigma.

Polymerase Chain reaction:

Polymerase Chain Reaction (PCR) is method developed by Kary Mullis in the 1980s. It is used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. PCR is based on utilizing the ability of DNA polymerase to synthesize new strands of DNA complementary to templates provided.

PCR Reaction Components:

INGREDIENTS	REACTION VOLUME
10X Reaction buffer	2.5µl
25mM dNTP	0.25µl
100µM Forward Primer	0.2µl
100µM Reverse Primer	0.2µl
Phusion polymerase	0.2 µl
100ngTemplate	1µl
Sterile distilled Water	19.9 µl
Total	25µl

PCR parameter used for amplification:

Step	Temperature(^{o}C)	Time	No. of Cycles
Initial Denaturation	95	3 mins	1
Denaturation	95	30sec	34
Annealing	55	30sec	
Extension	72	1min	
Final Extension	72	5 min	1

Agarose Gel Electrophoresis:

Agarose is a polysaccharide, generally extracted from certain red seaweed (*Gelidium, Gracilaria).* It is a linear polymer made up of repeating unit of agarobiose, which is a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose. Agarose Gel Electrophoresis separates DNA fragments by their size in the agarose gel matrix. DNA samples are pipetted into the sample wells. DNA fragments are separated by applying an electric field to negative (anode) end causes the negatively-charged DNA to migrate towards the bottom positive (cathode) end. The rate of migration is proportional to the size i.e. smaller fragments move faster than the larger fragments in gel. DNA is visualized by including in the gel an intercalating dye, ethidium bromide. DNA fragments take up the dye as they migrate through the gel. Illumination with ultraviolet light causes the intercalated dye to fluoresce. 1% gel was made using 1X TAE buffer (40mM Tris, 20mM Acetate and 1mM EDTA and typically has a pH around 8.6). After melting the agarose, cool it down to nearly bearable temperature Ethidium Bromide was added. After mixing pour into the Horizontal electrophoresis apparatus and cooled down. The amplified product was loaded into the wells and run the assembly.

Preparation of Competent Cells DH5α

Compositions

RF1 (100 ml)	**RF2(100 ml)**	**SOB Medium(100ml) pH-7.0**
RbCl : 1.2 Gms	MOPS : 2ml of 0.5M(pH-6.8)	Bacto Tryptone : 20gms
$MnCl_2.4H_2O$: 0.99Gms	RbCl : 120mg	Bacto YE : 5gms
CH_3COOK : 3ml of 1M	$CaCl_2$: 1.1gms	NaCl : 2ml of 5M
$CaCl_2.2H_2O$: 0.15gms Glycerol : 15.0gms	Glycerol : 15ml	KCl : 2.5ml of 1M

***All the operations were performed at 4°C unless otherwise mentioned**.

50ml of SOB medium was prepared and autoclaved. 1 ml of 1M $MgCl_2$ was added prior to addition of starter culture. After inoculation, the starter culture was kept in 37°C incubator and allowed to grow the cell till mid log phase. Then optical density (A=600nm) of the culture was measured. It was in between 0.6-0.8. The culture was then centrifuged at 5000 rpm for 30mins in Beckman Coulter cold centrifuge and supernatant was discarded. The pellet was then suspended in $1/3^{rd}$ volume of filter sterilised RF1 solution and incubate on ice for 30mins. Again it was centrifuged at 2500rpm for 30mins. The pellet was suspended in $1/8^{th}$ volume of filter sterilised RF2 solution and incubate on ice for 30mins. Finally competent cells were aliquoted 200µl each in 1.5ml click lock tube (eppendorf) and stored at -80°C refrigerator.

Preparation of 50X TAE Buffer Compositions (for 1Litre)

Tris : 242g
Glacial Acetic Acid: 57.1ml
EDTA : 18g

All the components were dissolved in MilliQ and pH was adjusted at 8. Each time before using the buffer 1X TAE buffer was prepared from the 50X stock.

Preparation of Vector and Insert

Insert

The mEGFP gene was PCR amplified from the mEGFP plasmid using specific forward and reverse primers, Taq DNA polymerase and 25mM $MgCl_2$. At the time of PCR denaturation temperature was maintained at 95°C for 3mins and 30 secs in a cycle, annealing temperature was maintained at 55°C for 30secs, and elongation temparature was maintained at 72°C for 1min in a cycle (since the amplified fragment of EGFP would be lower than 1Kb and the amplification rate of Taq DNA polymerase is 1Kb/min) and finally for 5mins before infinite hold. After that the size of the PCR product and amplification of the desire fragment was confirmed by agarose gel electrophoresis. The PCR product was again subjected to Agarose Gel Electrophoresis to excise the PCR amplified DNA fragment using a scalpel and to remove primers and template DNA. Gel extraction was done using DNA gel extraction kit according to standardise protocol to purify DNA from agarose gel. After extraction, the concentration of the PCR amplified fragment was determined from analytical gel.

Digestion of Insert with *HindIII/NotI*

PCR amplified EGFP DNA fragment was then digested by restriction enzymes *HindIII* 0.5 unit and *NotI* 2.0 unit. 40 units of 10X Tango buffer was used along with the 20 units of GFP PCR product. The whole mixture was incubated at 37°C water bath for 3Hrs.

HindIII restriction site

NotI restriction site

Digestion of vector pCDNA3.1 with *HindIII/NotI*

5µg (50µl of conc. 100ng/µl) of pCDNA3.1 vector was added to the 40µl of 10X Tango buffer and nuclease free H_2O. The mixture was divided in 2 eppendorfs marked *HindIII* and NotI. 0.5unit *HindIII* was added to one eppendorf and 2units *NotI* was added to another eppendrof and kept in the 37°C water bath for 1hr to linearise the supercoiled DNA by the restriction enzymes. After that the level of digestion was checked by 1% agarose gel electrophoresis. After confirmation of digestion alternative enzymes were added to both the tubes and again kept them for incubation. Finally the mixture was pooled into one tube and allowed to incubate at 37°C. After 3hrs of incubation, both the vector & insert were run on preparatory gel and respective DNAs were excised from agarose gel using a scalpel. The DNAs from agarose gel were purified using sure Extract Gel Extraction kit (Nucleopore). Their concentrations were determined from the analytical gel.

Ligation reaction

Two eppendorfs were marked as V and V+I. In V+I, typically 20ng of vector DNA and 10-fold molar excess of insert were used in a ligation reaction in a total volume of 20 µl. In an eppendorf (V) ligation mixture for only vector was prepared as a control. Both the eppndorf containing ligation mixture was incubated at 16°C overnight.

Ligation mixture calculation

$$\text{Insert amount (ng)} = \frac{\text{(Size of the Insert (bp), EGFP) X (ng of Vector DNA) X (fold in molar ratio)}}{\text{(Size of the Vector (bp) pcDNA3.1)}} \text{ ng}$$

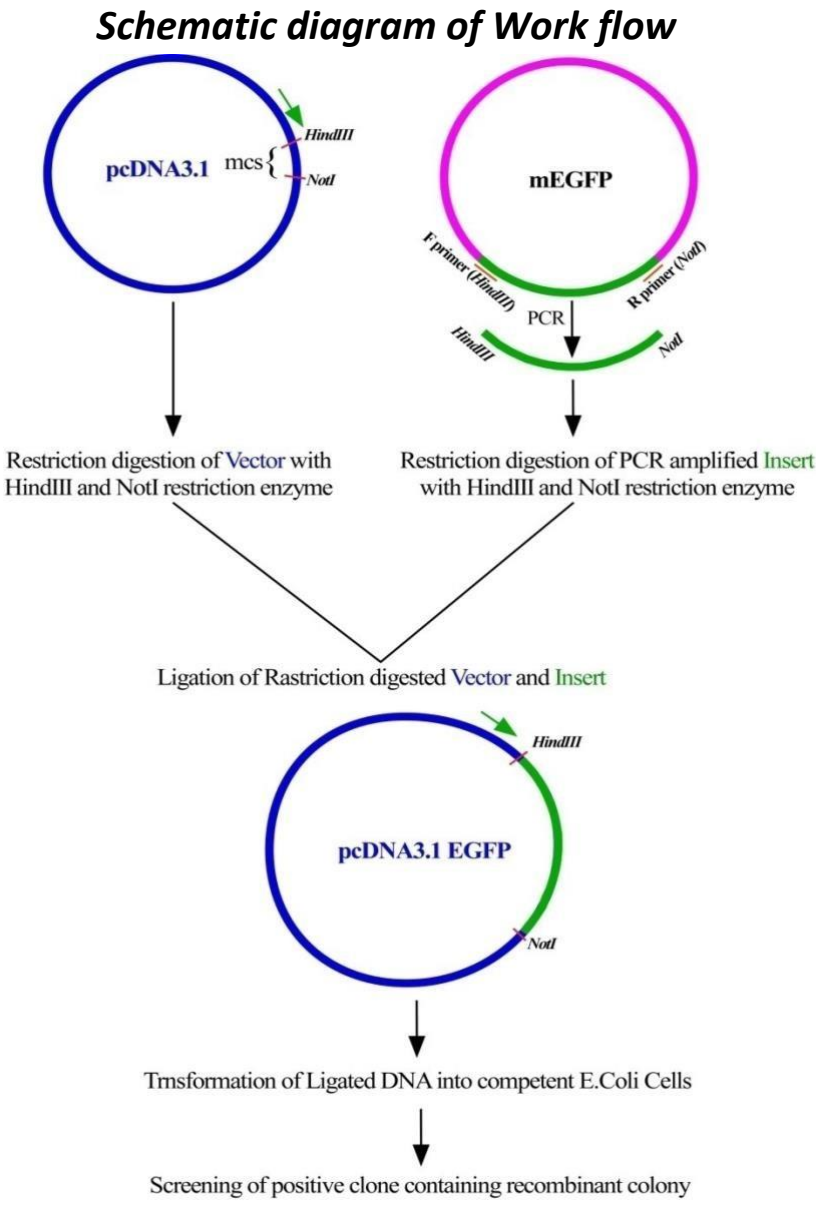
Bacterial transformation

DNA product from the ligation mixture was mixed with 200µl of DH5 competent cells. The tubes were incubated on ice for 30 minutes, heat shock was applied at 42°C for 2mins and then returned to ice for 2mins. 800µl Luria-Bertani (LB) medium was added to each eppendorfs and the tubes were placed in a shaker at 37°C for an hour. After incubation, the culture were spread onto LB agar plates containing appropriate antibiotic (ampicillin 100µg/ml) and incubated at 37°C overnight.

Screening of Recombinants by Colony PCR:

Around 12 colonies were picked up from the transformed plate and streaked on Amp + LB plate (replica plating) followed by transferred to 30 l of H_2O which was labelled appropriately. All the tubes were boiled

for 10 mins & then centrifuged at 10,000 rpm for 10mins.The replica plate was incubated at 37°C in incubator. Parallelly colony PCR was performed with mEGFP specific primers using 10µl of colony supernatant & mEGFP PCR product was used as positive control. To analyse the recombinants colony supernatant from the vector plate was used as negative control. The PCR products were then ran on 1% agarose gel to rule out negative samples. The colonies became positive through the gel electrophoresis were inoculated separately from the replica plate in 5ml LB containing falcons and kept in shaker incubator. From these cultures plasmid isolation was performed with Midi-prep plasmid isolation Kit (Nucleopore) using manufacturers protocol. The plasmids were again digested with HindIII and NotI (the same enzymes by which pCDNA3.1 and mEGFP inserts were digested) and subjected to agarose gel electrophoresis to check if the GFP has been inserted into pcDNA3.1 vector.



Results and Discussion

A) <u>Amplification of EGFP fragment:</u> mEGFP gene was amplified by PCR using mEGFP plasmid as template. The size of the PCR product was obtained from the gel electrophoresis was approximately **700Kb** according to molecular marker [***Fig1***]. After determining insert size, a preparatory gel was run to excise the PCR-GFP band and gel extraction was performed, concentration of purified mEGFP was obtained-**75mg/µl** by extraction of preparatory gel using sure Extract Gel Extraction kit (Nucleopore). After amplification of the insert gel extraction was done to remove primer because *HindIII/NotI* was introduced at the either site of the GFP gene sequence by using primers. If primers are not removed they may interfere with restriction enzymes *HindIII/NotI* at the time of digestion.

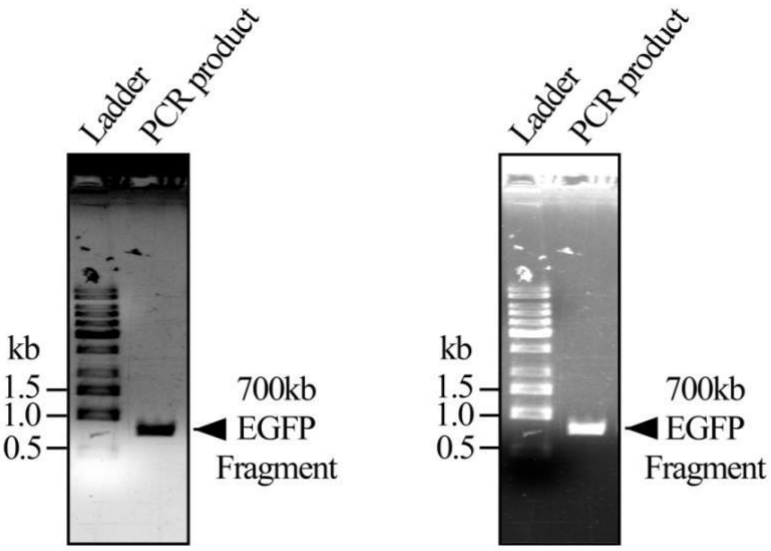
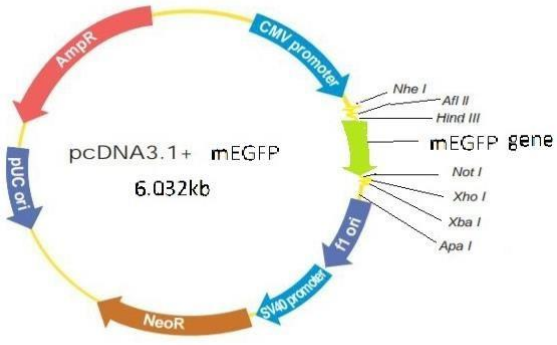


Fig1: PCR amplification of GFP DNA fragment: *PCR amplified EGFP fragments (~700b) using mEGFP plasmid as template. Lane 1 represents the DNA ladder and lane 2 represents the PCR amplified fragment. The right side figures is only the inverted image of the first one for better resolution*

B) <u>pcDNA3.1 Vector digestion:</u>

Traditional Cloning is the method of cloning in which we use restriction endonucleases to produce DNA fragments with specific complementary end sequences that can be joined together with a DNA ligase enzyme prior to the transformation. This technique involves preparing both the insert mEGFP and plasmid pCDNA3.1 in which we are going to ligate our gene of interest (EGFP), by digesting with two unique restriction enzymes (*HindIII/NotI*) that flank the DNA sequence on both sides with sticky ends, and whose cut sites are present at the preferred site of insertion of the vector, called the multiple cloning site (MCS).



Proposed map of the GFP cassette construct

By using two different REs (*HindIII* and *NotI*), two non-compatible ends are generated in the vector which were not ligated to itself, thus forcing the insert to be cloned directionally, and lowering the transformation background of re-ligated vector alone so this method is accurate at some extent. The enzymes are added alternatively, not at a time to ensure that each of them is working efficiently and independently.

According to the pCDNA3.1 (5.4kb) vector maps *HindIII* and *NotI* enzymes were chosen for digestion of the vector and insert respectively. Digestion of pCDNA3.1 plasmid with *HindIII* & *NotI* [***Fig2***] was expected to produce two fragments: 5.332 kb & 68 bp (which will be lost) the smallest fragment will be replaced with the 700bp mEGFP gene.

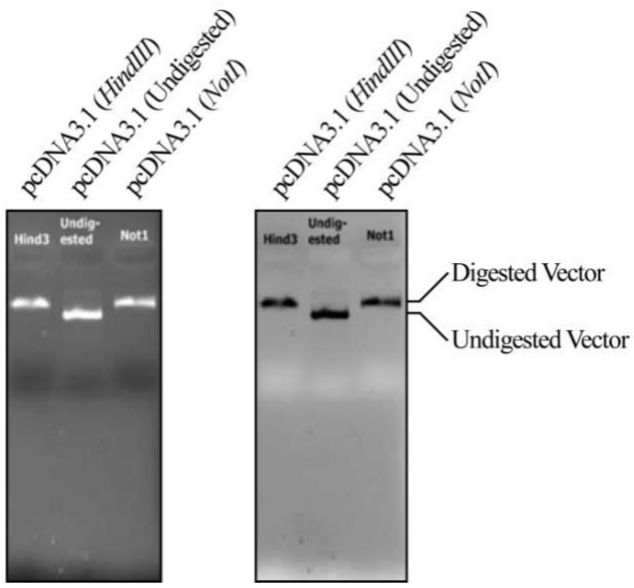


***Fig2**: **pCDNA3.1 empty vector**: pcDNA3.1 vector digested by only one restriction enzyme. Here in lane 1 with HindIII, Lane 2-control-undigested pcDNA3.1 and in lane 3withNotI. The digested vector has been linearized as migrated slowly in agarose gel electrophoresis as compared to undigested supercoiled control plasmid.*

After completion of the digestion gel extraction was done. Concentration of both the digested pCDNA3.1 and mEGFP were obtained from the analytical gel after gel extraction.

Concentration of pCDNA3.1 (*HindIII/NotI*): **20ng/□l**

Concentration of EGFP fragment (*HindIII/NotI*): **40ng/□l**

C) <u>pcDNA3.1 Vector and EGFP ligation mix transformation:</u>

In laboratory we used to use genetically modified *E. Coli* strain which does not contain any gene for antibiotic resistance so that transformed colony having antibiotic resistance plasmids can grow only. Here in pcDNA3.1 ampicillin resistant gene is present so that, only the transformed colony having pcDNA3.1 can grow on LB-Amp plate. A transformant is a cell that has taken up additional DNA—usually a plasmid that confers some kind of antibiotic resistance, so that successful transformants will grow, while all of the cells that weren't transformed will not.

Gel extracted digested vector and insert has been added in a 1:10 molar ratio along with the ligase enzyme (marked as V+I) in one eppendroff and in another digested vector was ligated along with ligase as a negative control to confirm the religation of only digested vector compared to the vector and insert vial. Then the ligated reaction mix has been transformed in precompetent *E.coli* cells and plated on the LB-Amp plate to get the transformed colonies. Here we have got more than 2000 colonies in vector insert mix transformed plate (V+I marked plate) whereas in only vector plate (V marked plate) we have got almost 500 colonies [***Fig3***].

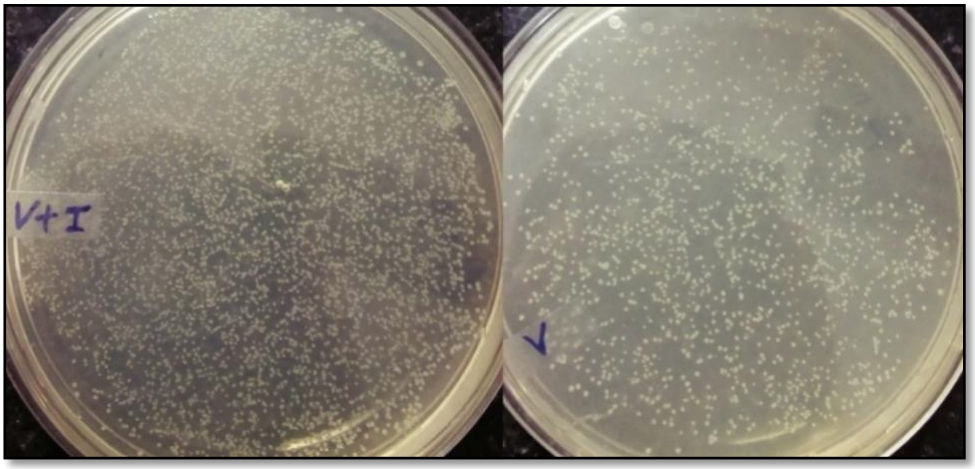


Fig3: Transformation of Ligation mix and colony counting: *The ligation mixture transformed and plated in LB-Amp agar plate. Here in plate (V+I) where we have transformed vector and insert ligation mix we have got more than 2000 colonies in contrast to 500 colonies in only vector reaction mix transformed plate (V).*

D) <u>Screening of recombinant colonies by colony PCR:</u>

When the insert mEGFP gene has been inserted in the vector successfully, the cells become recombinants. Only recombinants are PCR positive. The recombinants were verified by colony PCR with GFP specific primer. This was done to make sure about the insertion of the mEGFP gene into the vector and also to recheck whether the colonies which we select in colony PCR should be re amplified in the same amount or not. We have taken 11 colonies from the (V+I) plate and 1 colony from the (V) plate as a negative control and mEGFP plasmid as the template of positive control. We have found all the colonies from (V+I) plates to be recombinant and contained the desired fragment of EGFP as insert in pcDNA3.1 vector.

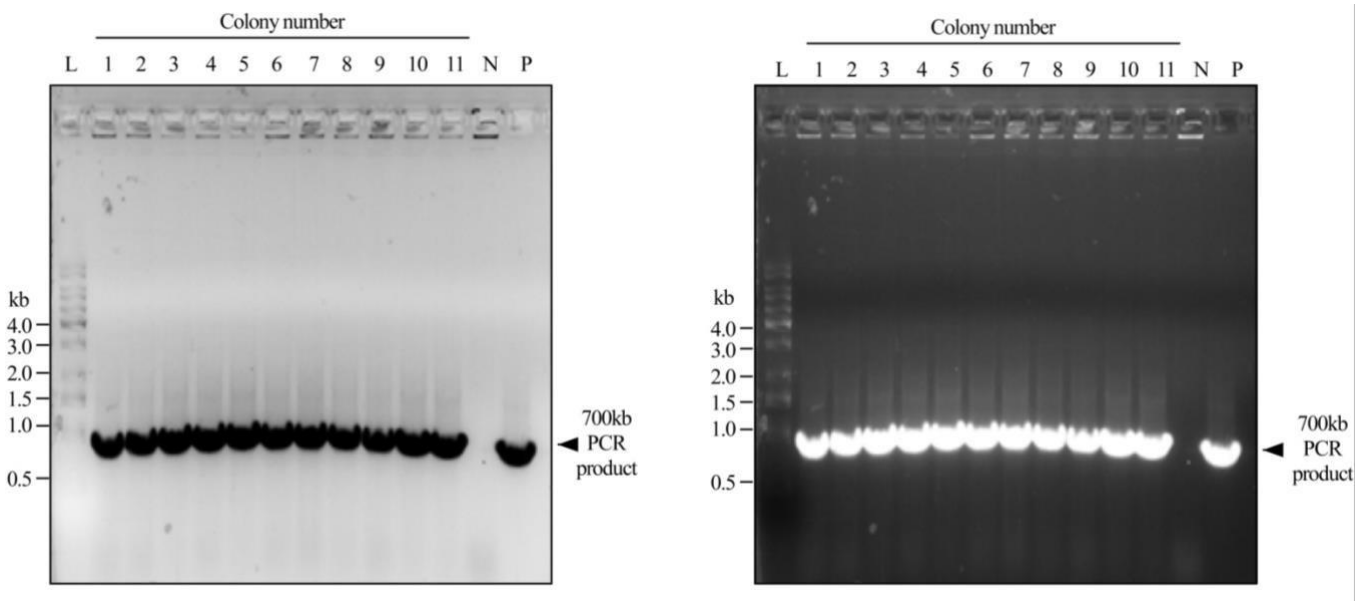


Fig4: Colony screening for the recombinant colony by colony PCR*: Here in this Agarose gel electrophoresis after colony PCR we have found that all the colonies taken from the V+I plates were found to be recombinant i.e they contained our desire fragment EGFP as insert. Here the numerical number represents the colony number, 'L' for DNA ladder, 'N' for negative control where we have added the colony from only vector plate (V), 'P' for positive control and in this case we have used mEGFP plasmid as the template for PCR amplification. 'kb' represents kilo base pair size of DNA ladder and right hand side image is the invertion of the left image for better resolution.*

E) <u>pcDNA3.1-EGFP clone confirmation by restriction digestion:</u>

From the agarose gel electrophosresis colony PCR image we have confirmed that all the colonies from V+I plate are recombinant. Therefore we have taken tow colonies and grow them in 3ml LB-Amp liquid broth for overnight (~16Hrs) at 37°C in incubator. The next day we have made glycerol stocks of those cultures and isolated the plasmid from those colonies. Next we digested the DNA isolated from those colonies with *HindIII* and *NotI* restriction enzymes to check the release of the insert. Here in case of the DNA from both the colonies i.e. for colony 1 and 2 we have found the release of the insert and its migration at its proper position (~700kb) in agarose gel electrophoresis.

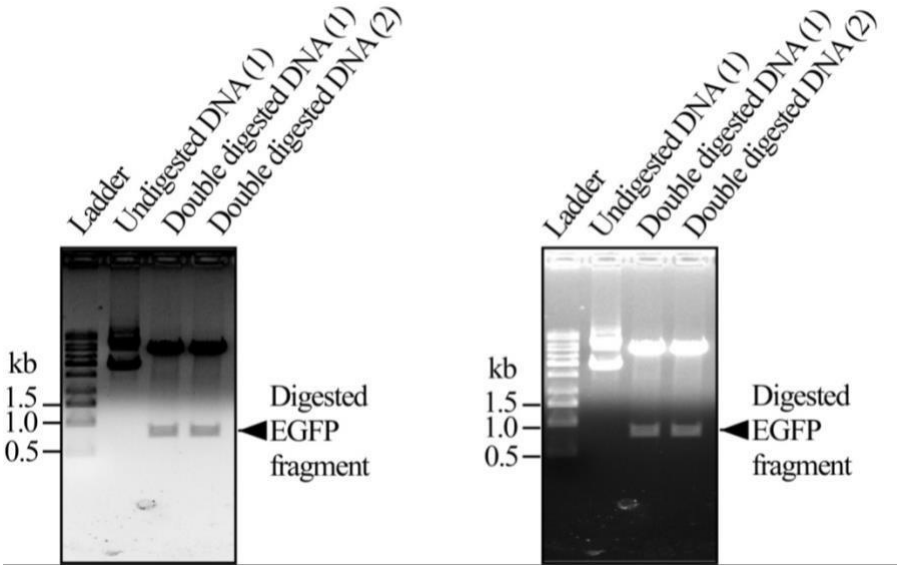


Fig5: Restriction digestion for clone confirmation: *Agarose gel electrophoresis after digestion of clone 1 & 2 with HindIII and NotI releases the desired EGFP fragments from pcDNA3.1 vector. Here numerical number 1 & 2 represents the DNA isolated from recombinant colony number 1 & 2. Lane 1-4 represents DNA ladder, undigested DNA from recombinant colony 1, double digested DNA from recombinant colony 1 & 2 respectively.*

These plasmids were further PCR amplified and stored for further checking the expression of the cassette construct. To systematize the results and prevent errors caused by mixing up of variables, the plasmids were analyzed using positive sampling and restriction digestion in all cloning experiments that conducted using the said samples. This was done to confirm that the insertions of the insert DNA into the sample vectors was done correctly. This is a highly delicate procedure. Therefore, minor errors during the procedure cannot be ruled out.

Conclusion and Future Prospects

This GFP-cassette construct can visualize particular cell types in whole animals, organs, tissues, and cell cultures. This possibility is particularly important in such fields as immunology, neurobiology, development, carcinogenesis etc. The greatest challenge to the broad application of GFP technology in drug discovery is that of sensitivity. The cassette can be applied in a large variety of studies related to various aspects of living systems, and the range of its applications is continuously expanding.

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