

Producing a Mammalian GFP Expression Vector Containing Neomycin Resistance Gene

Manizheh Izadi, Maryam Abiri, and Mohammad Keramatipour *

Department of Medical Genetics, Faculty of Medicine, Tehran University of Medical Science, Tehran, Iran

Abstract

The green fluorescent protein (GFP) was originally isolated from the Jellyfish *Aequorea Victoria* that fluoresces green when exposed to blue light. GFP protein is composed of 238 amino acids with the molecular mass of 26.9 kD. The GFP gene is frequently used in cellular and molecular biology as a reporter gene. To date, many bacterial, yeast, fungal, plants, fly and mammalian cells, including human, have been created which express GFP. Martin Chalfie, Osamu Shimomura, and Roger Tsien were awarded the 2008 noble prize in chemistry for their discovery and development of GFP. In many studies on mammalian cells, GFP gene is introduced into cells using vector-based systems or a recombinant virus to track the location of a target protein or to study the expression level of the gene of interest, but in these studies there is no selection marker to normalize transfection. According to the importance of neomycin gene as a selection marker in mammalian cells, we aimed to produce a GFP expression vector that contains neomycin gene. GFP gene was separated from pEGFP-N1 vector and was inserted in the backbone of pCDNA3.1/His/LacZ vector that contained the neomycin gene. The resulted vector contained GFP beside neomycin gene.

*Corresponding Author:

Mohammad Keramatipour,
M.D., Ph.D., Department of
Medical Genetics, Faculty of
Medicine, Tehran University
of Medical Science, Tehran,
Iran, P.O. Box: 14155-6447

Tel: +98 21 88973655

Fax: + 98 21 88953005

E-mail:

keramatipour@tums.ac.ir

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Introduction

In the 1960s and 1970s GFP along with the separate luminescent protein Aequorin, was purified from *Aequorea Victoria* and was characterized by Osamu Shimomura^(1, 2). Its ability as a tool for molecular biology research was recognized in 1992, when Douglas Prasher cloned wtGFP and reported its nucleotide sequence⁽³⁾. The first reported crystal structure of GFP was that of the S65T mutant by Remington group⁽⁴⁾. One month later, the wild type GFP structure was described in Nature Biothec⁽⁵⁾.

The availability of GFP and its derivatives has thoroughly redefined fluorescence microscopy and the way it is used in cell biology and other biological disciplines^(6, 7). 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.

In such cases, GFP gene is inserted into the genome of organism in the region of DNA which codes for the target proteins, and is controlled by the same regulatory sequence. In cells where the gene is expressed and the tagged proteins are produced, GFP is produced at the same time^(7, 8).

Therefore, only those cells in which the tagged gene is expressed, will fluoresce when observed under fluorescent microscope. Analysis of such time lapse movies has redefined the understanding of many biological processes such as protein folding, protein transport and RNA dynamics, which in the past had been studied using fixed material. Another 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⁽⁹⁾.

Materials and Methods

Vectors

pEGFP-N1 (Clontech Laboratories, Inc. GenBank Accession #U55762) vector containing GFP gene, and pCDNA3.1/ His/ LacZ vector (Invitrogen life technologies) which includes neomycin resistant gene, were used in these experiments.

In all cloning experiments, the vector was cut by the appropriate restriction enzyme and run on a 1% agarose gel. Linear vector was extracted from the gel using Fermentas DNA extraction from agarose gel kit.

To eliminate the background recircularisation,

vectors with compatible ends were dephosphorylated. 3-5 μ g of vector was incubated with 20 *units* of calf intestine alkaline phosphatase (CIAP, from Amersham Pharmacia Biotech) at 37°C for 30 *minutes*.

Dephosphorylated vector was purified using the Fermentas PCR purification kit and its concentration measured by Spectrophotometry.

Inserts

The DNA fragments to be cloned were produced by restriction digestion of the source vector. Products were extracted from 1% agarose gel using QIA quick gel extraction kit (Qiagen) and their concentrations were determined by Spectrophotometry.

Ligation reaction

Typically 100 *ng* of vector DNA and a 2 to 5-fold molar excess of insert were used with 200 *units* of T4 DNA ligase (New England Bio labs) in a total volume of 10 μ l at 16°C or 4°C (according to the recommendation of enzyme manufacturer) for 16 *hours*. 1 μ l of the ligation mixture was then used to transform Top10F competent cells.

Bacterial transformation

1 μ l of DNA product from the ligation mixture was mixed with 50 μ l of Top 10F competent cells. The tubes were incubated on ice for 30 *minutes*, heat shocked at 42°C for 60-90 *seconds* and then returned to ice. After

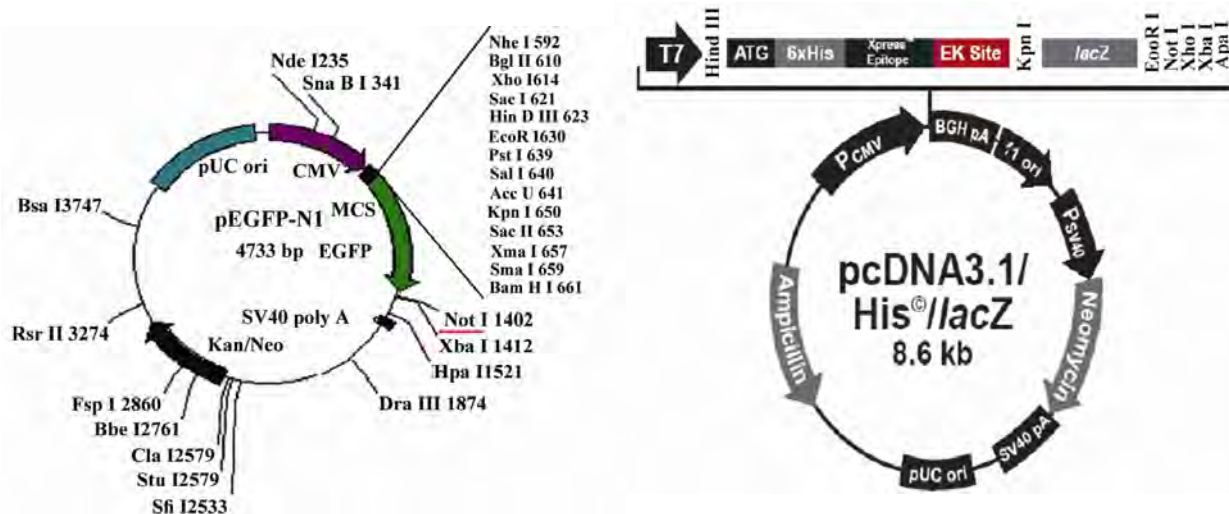


Figure 1. Vector maps of pEGFP-N1 and pcDNA3.1/His/LacZ

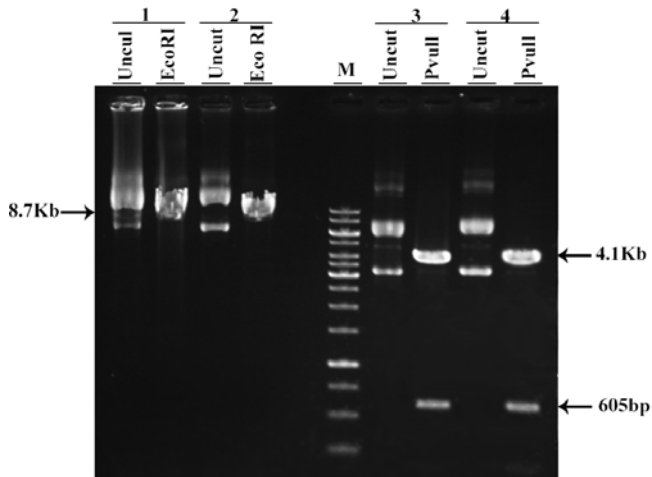


Figure 2. Restriction analysis of pEGFP-N1 and pCDNA3.1/His/LacZ
 1&2: different clones of pEGFP-N1
 3& 4: different clones of pCDNA3.1/His/LacZ
 Uncut: undigested plasmids
 M: 1 Kb marker

two minutes, 950 μ l Luria-Bertani (LB) medium was added to each transformation and the tubes were placed in an orbital incubator at 37°C for 1 hour, 250 RPM. Aliquots of 100 μ l of the culture were spread onto LB plates containing appropriate antibiotic (kanamycin 50 mg/ml or ampicillin 100 μ g/ml) and incubated at 37°C overnight.

Plasmid DNA preparation

4.5 ml of LB solution containing ampicillin (100 μ g/ml) was inoculated with a single colony and the suspension was grown at 37°C overnight. DNA was prepared from a 4 ml culture using the Accuprep Plasmid Extraction Kit (BioNeer, Korea).

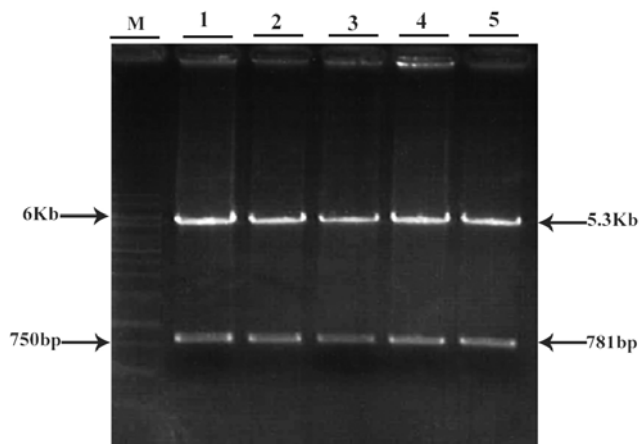


Figure 4. Restriction analysis of GFP/Neo vector
 1-5: different clones of GFP/Neo vector after double digestion with *HindIII/NotI*

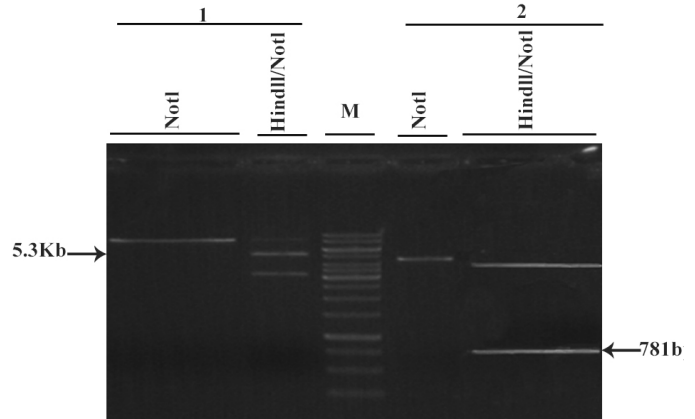


Figure 3. Extraction of GFP and Neomycin fragments after *HindIII/NotI* double digestion
 1: pcDNA3.1/His/LacZ
 2: pEGFP-N1
NotI: linearized vector with *NotI*
 781 bp: GFP fragment of pEGFP-N1 after double digestion with *HindIII/NotI*
 5.3 Kb: Back-bone of pcDNA3.1/His/LacZ after double digestion with *HindIII/NotI*
 M: 1 Kb marker

In all cloning experiments the plasmids were analysed by restriction digestion and positive samples were sequenced to confirm the correct insertion of insert DNA into the vector. Sequencing was performed by Genfanavaran Company, Iran.

Results

After transformation of *E. coli* Top10F strain cells with pEGFP-N1 vector and pCDNA3.1/His/LacZ vector, a number of obtained colonies were screened using restriction enzyme digestion. According to the pEGFP-N1 and pCDNA3.1/His/LacZ vector maps (Figure 1), *PvuII* and *EcoRI* enzymes (Fermentas) were chosen for digestion of these vectors, respectively. Digestion of pEGFP-N1 plasmids with *PvuII* was expected to produce two fragments: 4.1 kb & 605 bp. And 8.7 kb band for linearized plasmid, was expected after digestion of pCDNA3.1/His/LacZ plasmids with *EcoRI* (Figure 2).

Double digestion of pEGFP-N1 vector by *HindIII* and *NotI* (Fermentas) resulted in two fragments (Figure 3).

The 781 bp fragment which contains GFP was purified from agarose gel. Double digestion of pCDNA3.1/His/LacZ vector by

HindIII and NotI (Fermentas) also produced two fragments (Figure 3).

The 5.353 *bp* fragment (the back-bone of the vector) containing neomycin resistant gene was purified from agarose gel. These two purified fragments were ligated and the ligation mixture was subsequently used for transformation of E.coli TOP10F cells. The resulted clones were screened by double digestion with HindIII and NotI. Those clones that produced two 5.3 *kb* and 781 *bp* bands after digestion were selected (Figure 4) and further analyzed by DNA sequencing. One of the confirmed clones was used for the sub-cloning phase.

Discussion

In this study we produced a new vector for GFP expression that contains neomycin resistant gene. In the absence of a selection marker, neomycin gene in this case, the level of transfection using GFP expression vector would be different in various wells. By using this vector (GFP/Neo) transfection could be normalized. After 48 *hours* of transfection, adding G418 antibiotic can be used to select only cells with GFP expression and remove cells lacking this vector; which is synonymous with transfection normalization.

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