Cloning, Expression and Purification of His-tagged Firefly Luciferase

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Introduction

Firefly luciferase is the enzyme responsible for the phenomenon of bioluminescence in insects such as firefly (*Photinus pyralis*), glowworm beetles and click beetles. Luciferase catalyzes the oxidation of luciferin resulting in the emission of yellow light 550nm to 620nm. Firefly luciferase is a 62kDa protein whose structure reveals a protein with two domains N and C. The active site lies in the C terminal domain. The large N terminal domain is made up of a β barrel and an αβαβα structure. The C-terminus has a fold called the α+β fold found in many hydrolases. The enzyme belongs to a superfamily of adenylate forming enzymes which depend on ATP in the adenylation reaction[1]. The goal of the project is to generate a recombinant DNA construct of a His-tagged luciferase gene in a pET expression vector, overexpress Luciferase in *E.coli* and purify the protein using Metal-Chelate Affinity purification.

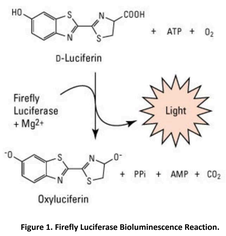


Figure 1. Firefly Luciferase Bioluminescence Reaction.

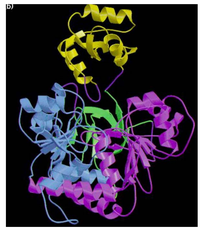


Figure 2. Firefly luciferase structure-Yellow is the smaller C term domain and the remaining is N term[1].

Experimental Design

In order to facilitate proper insertion of the luciferase coding sequence the following primer pair was designed for PCR:

Forward primer with a BamH1 restriction site (green):

GAGGATCC**ATG**GAAGACGCCAAAAAC

Reverse primer with a Sac1 restriction sites (green):

GTGAGCTCTTACAATTTGGACTTTCCG

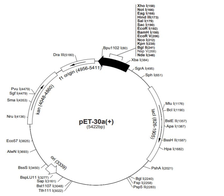


Figure 3. Vector map of pET-30a

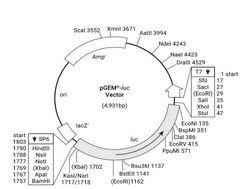
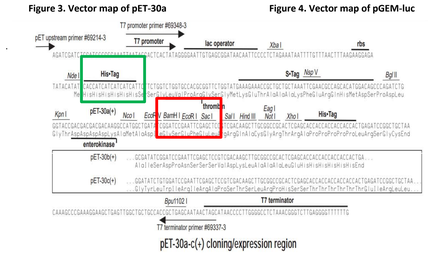


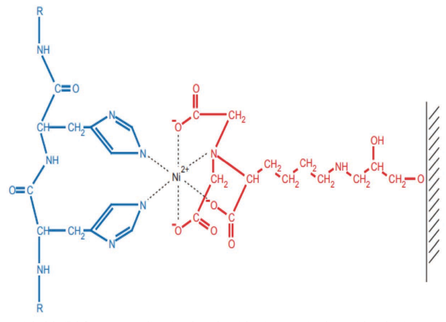
Figure 4. Vector map of pGEM-luc



Experimental Methods

Polymerase chain reaction (PCR) was utilized to amplify the luciferase gene found in pGEM-luc using Forward and Reverse primers to introduce restriction sites for BamH1 and Sac1 on either sides of the coding sequence of luciferase. The luciferase PCR fragment and the pET30a vector were digested using BamH1 and Sac1. The digested luciferase fragment and linearized pET30a were gel purified and subsequently ligated using T4 DNA ligase. Bacterial transformation (heat shock at 42°C for 1 min) was done with control and ligated recombinant plasmids using DH5α cells plated on LB agar media with kanamycin antibiotic. After overnight incubation plasmid purification (GeneJET Miniprep kit) was performed to purify recombinant plasmid DNA. A diagnostic digest was performed to identify the recombinant pET30a carrying luciferase (pET30a-luc). pET30a-luc plasmids were then transformed into BL-21 DE3 *E. coli* cells, which are ideal for overexpression of the protein. After overnight incubation of the BL-21 DE3 cells, colonies were picked and grown overnight in 4mL liquid culture. They were then transferred to 250mL LB agar media with kanamycin and grown until an optical density (OD) of 0.6 was reached. Expression of luciferase was done through fast induction by incubating cells with IPTG (final concentration 1mM) for 3 hours. After fast induction, the liquid culture was centrifuged for 5 min at 7000rpm in 4°C and the pellet bacterial cells were stored at -20°C.

Immobilized Metal Affinity Chromatography

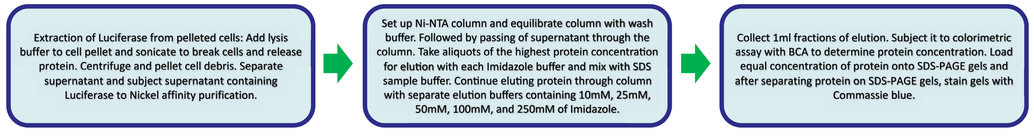


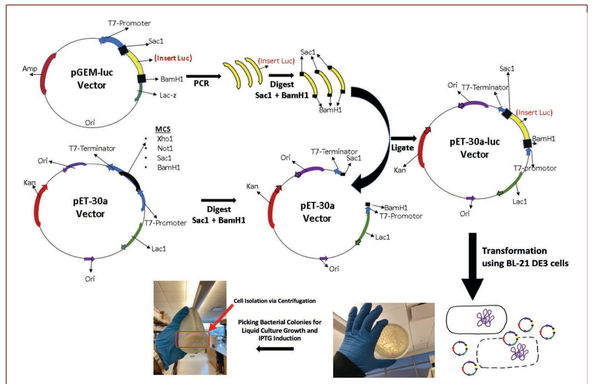
Nickel (Ni^{2+}) forms coordination bonds with Nitrogen in the Imidazole ring of Histidine. This is how the Histidine in the poly-His tag bind (Ni^{2+}) ions on the Ni-NTA agarose column.

Immobilized Metal Affinity Chromatography

Immobilized Metal Affinity Chromatography (IMAC) is a commonly used versatile protein purification technique that can yield a very high purity protein. The technique is commonly used to purify recombinant proteins carrying a polyhistidine tag. IMAC uses columns filled with matrices which have a transition metal such as Ni^{2+} or Co^{2+} immobilized on the matrix. The electron donor groups in the side chain of Histidine form coordination bonds with the transition metal. This therefore binds the recombinant protein on the column and all other proteins are washed out by a wash buffer. Eventually the protein can be eluted from the column by using increasing concentrations of Imidazole in the elution buffer. Imidazole has the same structure as the side chain of Histidine and hence competes with the Histidine for the NI^{2+} ions.

Immobilized Metal Affinity Chromatography Protocol





The pET30a vector, a strong expression vector, consists of a T7 promoter, N and C-terminal Histidine (His) tags, transcription initiation site, ribosome binding site for translation and are IPTG (Isopropyl β-d-1-thiogalactopyranoside) inducible. The lac I repressor expressed by the pET vector will bind the lac operator and block gene expression. IPTG can block the repressor and enable expression of the genes under the control of the T7 promoter. IPTG allows for proper expression of His-tagged proteins which can be purified with affinity chromatography. The luciferase gene was sourced from pGEM-luc (Promega).

Results

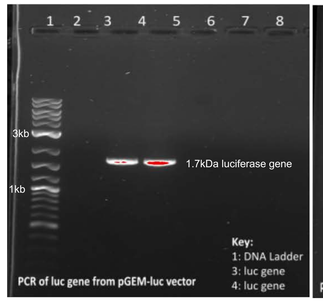


Figure A. Gel electrophoresis image of the luc gene from pGEM-luc vector

Figure B. Gel electrophoresis image of the pET30a-luc Construct Diagnostic Digest using Restriction Enzymes NotI and EcoRI

A diagnostic digest, which allows for analysis of plasmid fragments, was done using restriction enzymes NotI and EcoRI to identify and compare the pET30a-luc samples to the control pET30a and pGEM-luc vectors. Figure A provides a gel electrophoresis image of the luc gene amplified by PCR of pGEM-luc. Figure B is a gel electrophoresis image in which 4 pET30a-luc samples were run and identified alongside a pET30a control and pGEM-luc sample. Band sizes of the fragments allowed us to confirm a successful ligation, in which we inserted the 1.7kb luciferase gene from the pGEM-luc vector into the pET30a vector. Lane 5 represents a diagnostic digest of pGEM-luc which resulted in 3 fragments of the following size: 3183kb, 1133kb, 615kb. Lane 8 represents a diagnostic digest of the control pET30a which resulted in a single band of 5396bp. Lastly, the successful ligation can be confirmed by observing the pET30a-luc samples in Lanes 2,3,4,6, and 7 which fragmented into two bands which align with the ~5kb of the pET30a control and the center ~1kb luc band from the pGEM-luc vector.

Next Steps

We will carry out affinity purification of His-tagged luciferase using a Ni^{2+} affinity column. We will repeat the process of expression using a slow induction method (18hrs with IPTG) and carry out the purification. This will enable us to make a comparison between both approaches for His-tagged Luciferase expression and purification.

Acknowledgements

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References: 1) Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes, https://doi.org/10.1016/S0969-2126(96)00033-0