Designing A Biosensor For Small Molecules In Bacterial System

Introduction

To create recombinant DNA for this investigation, a modified PQE60 plasmid that was first transformed with a GFP gene was genetically modified and added to the DH5 Alpha strain of E. coli. The EutS and EutR genes were introduced to this plasmid. E. coli was chosen as the model organism for these studies because it is known to exhibit the EutS signaling pathway. The method of experimentation is cultivating DH5 Alpha E. Coli.

Aim

To create a vitamin B12 sensor by cloning or genetically modifying an E. coli strain. This was accomplished by inserting a PQE60 GFP plasmid into an mt DH5 Alpha E. Coli strain.

Construct Design

The eut operon's sequence of genetic events in the ethanolamine utilization pathway gives the cell a particular way to detect and react to the presence of vitamin B12 and ethanolamine. Thus, the eutS gene and EutS protein can be utilized as fundamental building blocks for the development of a genetic biosensor for vitamin B12.

The EutR gene is translated into the EutR protein, a transcription factor that is crucial to the ethanolamine utilization regulatory network. When the EutR protein in cells comes into contact with its substrate, ethanolamine, together with vitamin B12, it forms a complex known as the EutR protein-Ethanolamine-Vitamin B12 complex. This assembly functions as a molecular switch to turn on the eutS gene's expression. The production of the EutS protein results from the activation of the EutS gene, and this ultimately enables cells to use ethanolamine.

The eutR and peutS genes would be the gene of interest and would be put into a plasmid vector to create the genetic biosensor. PQE60 GFP is the plasmid vector that was selected for this use; it was obtained from an IISC scientist and was already altered to carry the GFP gene.

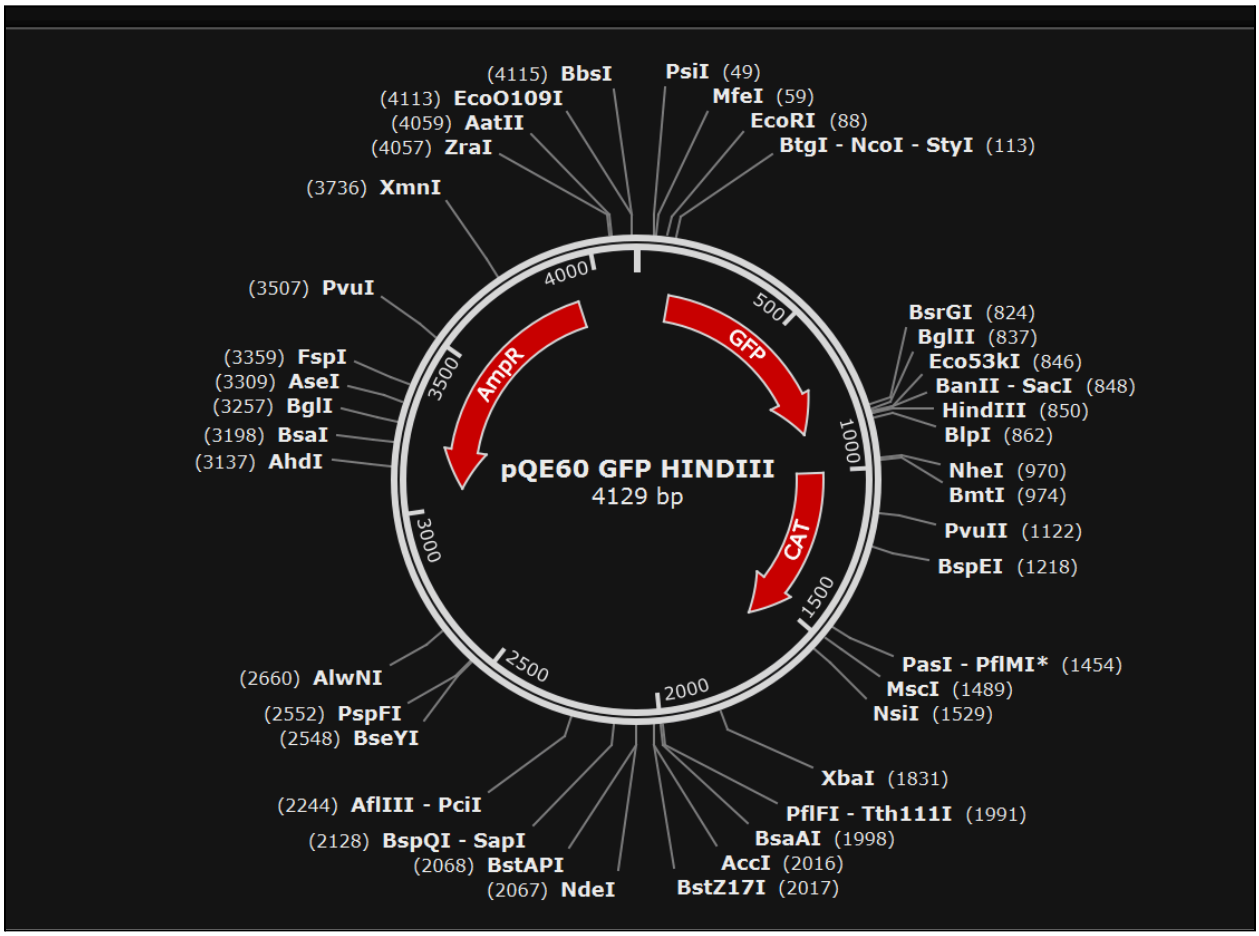
After selecting the vector and the desired gene, an insilico construct was created on Snapgene, which led to the construction of the primers that would be utilized for amplification and gene insertion into the plasmid vector.

Decide Gene of Interest:

In this work, a modified PQE60 plasmid, initially transformed with a GFP gene, was genetically modified to produce recombinant DNA in the DH5 Alpha strain of E. coli. The **peutS and eutR** genes were introduced to this plasmid. E. coli was chosen as the model organism for these studies because it is known to exhibit the eutS signaling pathway. The method of experimentation is cultivating DH5 Alpha E. Coli.

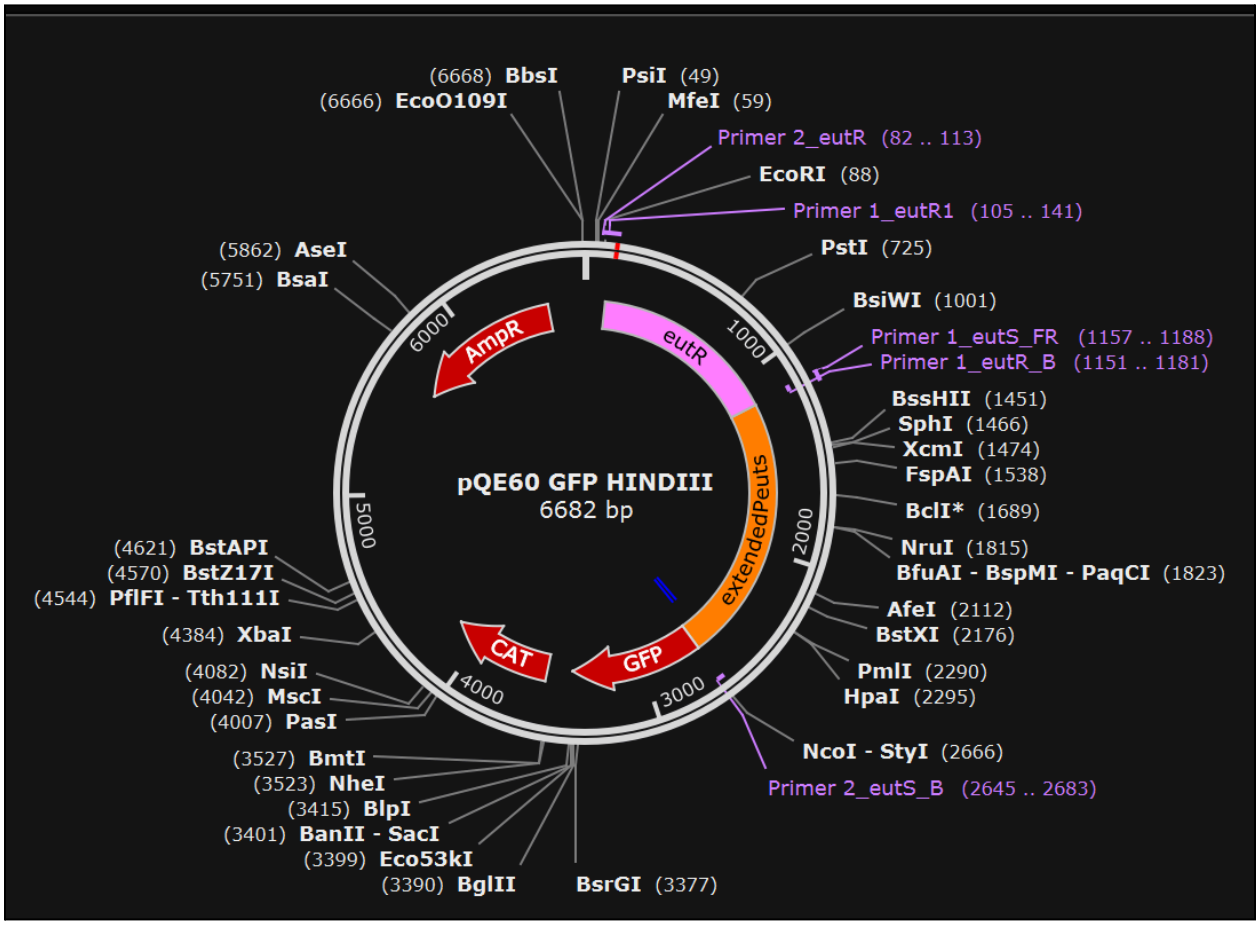
The goal is to create a vitamin B12 sensor by cloning or genetically modifying a strain of E. coli. To do this, an mt DH5 Alpha E. coli strain was injected with a PQE60 GFP plasmid.`

Decide Vector:



pQE60 GFP Hindiii.dna

Insilico construction:



Insilico construct Map of PQE60 GFP, peutS and eutR

Design Primers:

Primer 1_eutR_B	CCACTTCGCTCTGTTtcacccccactcccgc	31-mer
Primer 1_eutR1	GAAATTAACatgaaaaagacccgtacagccaatttgc	37-mer
Primer 1_eutS_FR	gtgggggtgaAACAGAGCGAAGTGGTGGCTTC	32-mer
Primer 2_eutR	CACACAGAATTCATTAAAGAGGAGAAATTAAC	32-mer
Primer 2_eutS_B	CGCCCTTGCTCACCATGGAGTCGCCGCCTGTGAAAAACT	39-mer

Primers from pQE60 eutR peuts GFP

Obtain Gene of Interest

Gene synthesis

- We use this method when the Gene sequence is unknown, as we know the sequence to use PCR.

PCR

- eutS
- eutR
- Amplified eutS eutR using PCR are known as primary PCR products.
- eutS + eutR(Primary PCR product was used again in PCR to make it into a single insert)
    - Troubleshoot when combined PCR is done it was not amplified because the region we chose was not in particular to the DH5 alpha in nature while we designed primer we used the snapgene file to generate premier but when we checked for the DH5 alpha strain of E coil it is not the actual sequence from the snapgene sequence besacially it was not matching with the primer designed with DH5 Alpha.

Cell Lysate PCR

Independent amplification of eutR and peutS genes using the designed primers:

- eutR and eutS genes are naturally present in the E. coli genome. So, to insert these genes it should first be isolated and amplified and then inserted into the vector.
- For that, Resuspend one colony from the DH5 Alpha E.coli cells agar plate in 20 µL of distilled water in a PCR tube
- Heat shock the suspension at 98°C to break the cell membrane and release the cellular components (used the thermal cycler to lyse the cell and remove the DNA)
- Centrifuge/spin down
- Discard the pellet and use the supernatant as a DNA template for PCR.

PCR protocol:

- Thaw the PCR master mix at room temperature
- Vortex the master mix then spin it down briefly in a microcentrifuge to collect the material at the bottom of the tube.
- For a 25 µL of PCR reaction:

Components	Amount to be added	Final concentration
PCR master mix 2X	12.5 µL	1X
Upstream primer 10 µM	0.25 - 2.5 µL	0.1 - 1.0 µM
Downstream primer 10 µM	0.25 - 2.5 µL	0.1 - 1.0 µM
Template DNA	1 - 5 µL	< 250 ng

Molecular biology PCR grade water	Add to make the total volume up to 25 µL	-

Making the stock solution of primers

Primers	Volume of water to be added to get 100pm/µL or 100 µL concentration
eutR_CFW1	238.9 µL
eutR_CFW2	311.82 µL
eutR_CRE	274.9 µL
eutS_CFW	338.7 µL
eutS_CRE	336.36 µL

These primer solutions were then diluted to make for 25 µL of PCR reaction.

PCR Mixture prep:

- Add 12.5 µL of master in a PCR tube
- Add upstream primer (2 µL for eutS and 4 µL for eutR)
- Add downstream primers (2 µL)
- Add template DNA of 1 µL
- Add PCR-grade water to make the total volume up to 25 µL

Two different PCR reactions were conducted for eutS and eutR separately, with two replicates. This was because the annealing and extension temperatures for the two gene amplifications were different.

Setting the PCR:

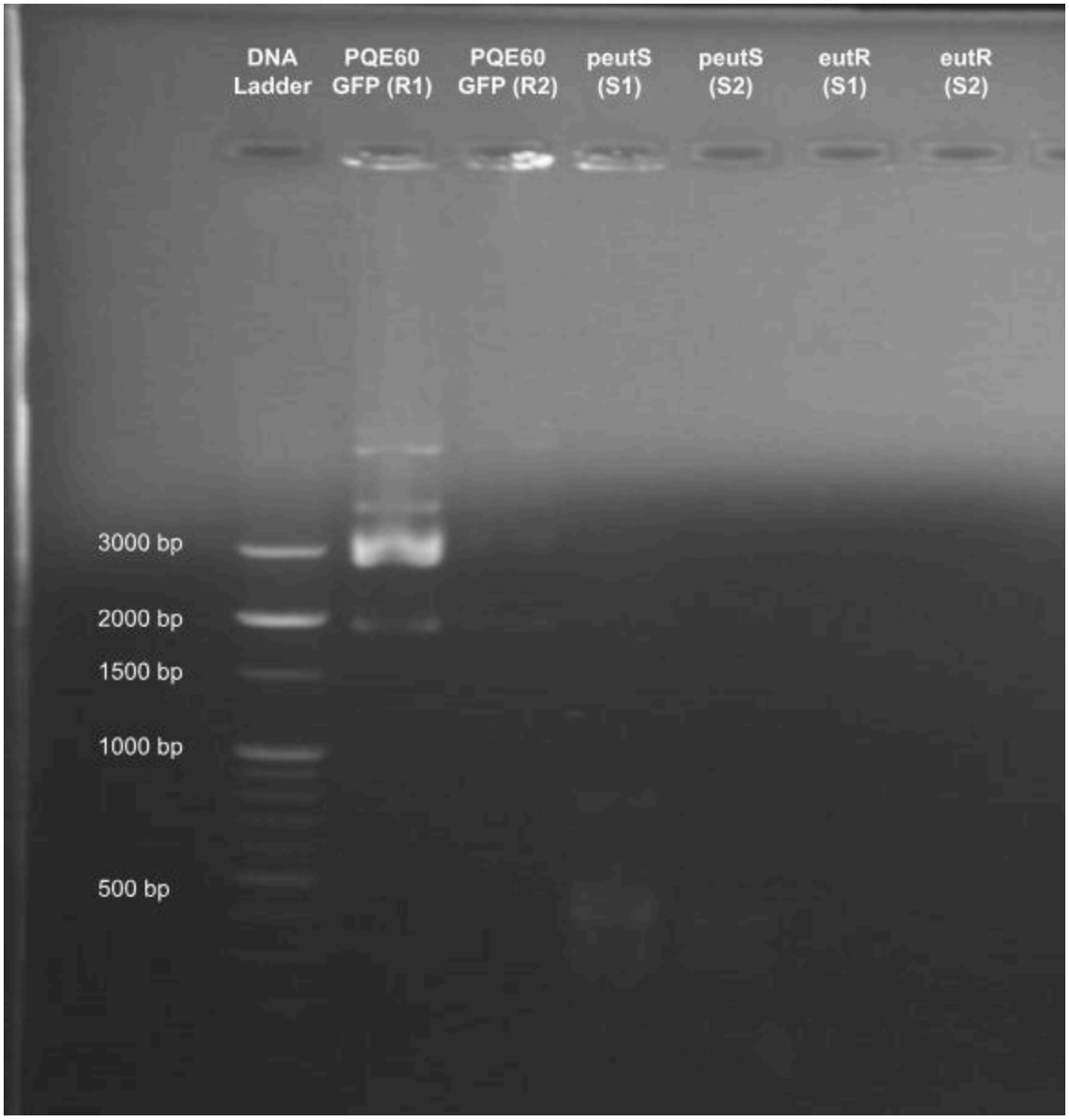
eutS

Step	Temperature (°C)	Time (min)
Initial denaturation	95	3:00
Denaturation	95	0:30

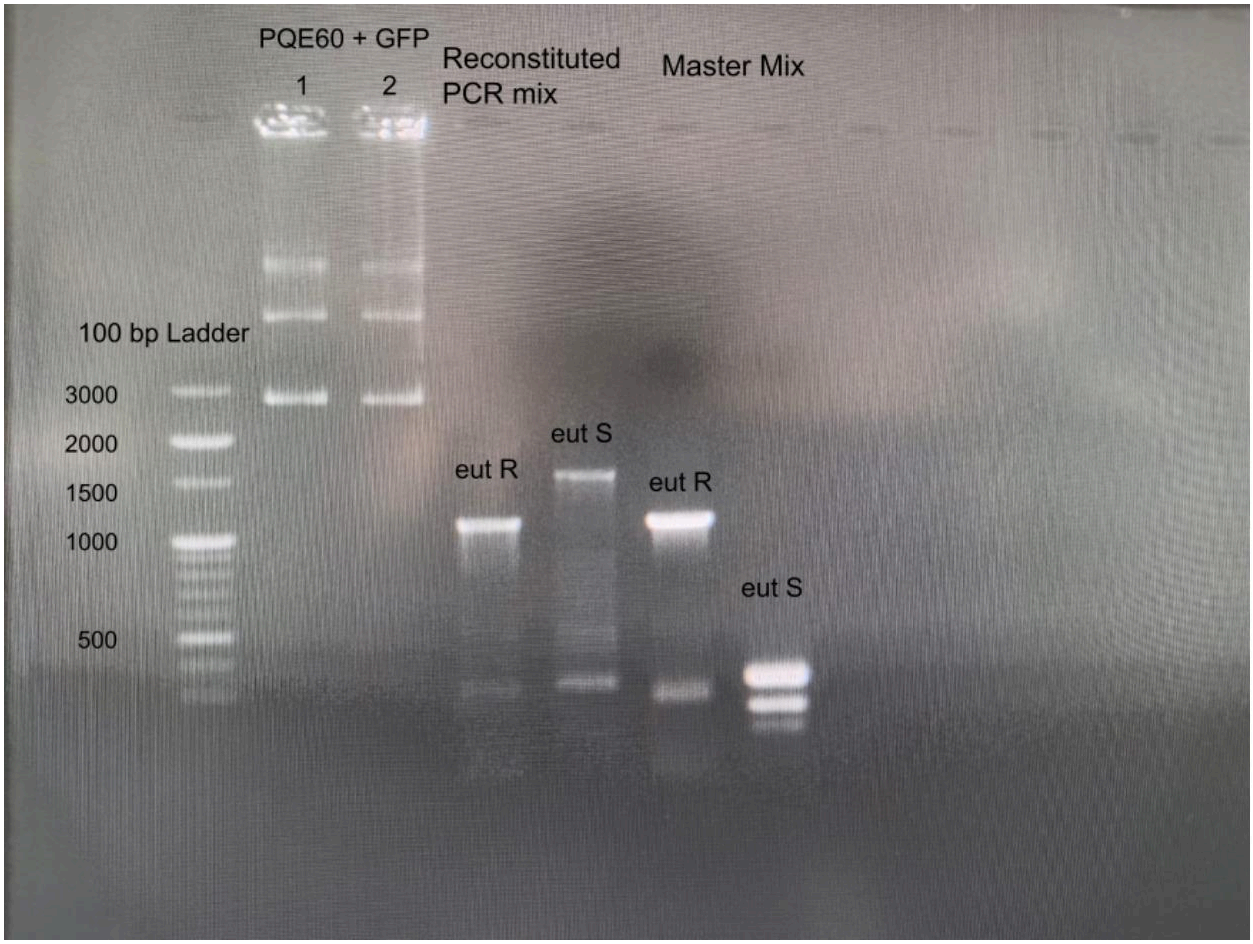
Annealing	60	0:30
Extension	72	1:30
30 cycles		
Final extension	72	5:00
Hold	12	Infinity
Total time for PCR =		1:48:27

eutR

Step	Temperature (°C)	Time (min)
Initial denaturation	95	3:00
Denaturation	95	0:30
Annealing	60	0:30
Extension	72	1:02
30 cycles		
Final extension	72	5:00
Hold	12	Infinity



The First PCR was a failed.



Observation

Sample	Main Band Observed (bp)	Expected Length (bp)
Plasmid (PQE60)	3000	3000
Reconstituted PCR Mix - eut R	≈1053	1053
Reconstituted PCR Mix - peut S	≈1500	1500
Master Mix - eut R	≈1053	1053
Master Mix - peut S	≈ 300	1500

The plasmid (PQE60) exhibits an observed main band matching its expected length of 3000 base pairs (bp), indicating intact DNA. Likewise, PCR mixes targeting the "eut R" and "peut S" genes show main

bands close to their expected lengths (≈1053 bp and ≈1500 bp, respectively), suggesting successful amplification. However, a discrepancy arises in the "peut S" gene PCR using the "Master Mix," where the observed main band (~300 bp) differs significantly from the expected size (1500 bp), indicating potential issues like incomplete or nonspecific amplification. There is nonspecific amplification at ~300 bp in every sample.

Prepare Vector

PQE60 Plasmid Isolation

- Grow E. coli DH5 Alpha strain and TG1 transformed with the plasmid of interest in 3 mL LB medium containing appropriate antibiotics in a tube After overnight (15–16 h) growth at 37°C, harvest the bacterial cells by centrifugation at top speed for 5 minutes at 5000 RPM at room temperature in a microcentrifuge.
- Resuspend the bacterial pellets completely in 1.5 mL ice-cold GTE (50mM glucose, 25mM Tris-Hcl, and 10mM EDTA of pH 8) by putting a toothpick in the tube and then vortex mixing for several seconds.
- Remove the toothpick and add 2.5 mL of freshly prepared lysis buffer (0.2 M NaOH, 1% sodium dodecyl sulfate [SDS]); mix quickly by inverting and swirling the tube several times until the contents become clear. Do not vortex.
- Precipitate cellular debris by adding 3 mL ice-cold 4 M potassium acetate solution (prepared by mixing 80 mL 5 M potassium acetate, 11.5 µL glacial acetic acid, and 8.5 mL H2O). Mix the contents thoroughly by immediately swirling the tube. Do not vortex mix.
- Centrifuge at top speed for 10 min and 10,000 RPM at room temperature and transfer the supernatant into a clean tube with a disposable pipet. Avoid collecting a floating film of denatured material by submerging the pipet tip well into the solution before suction.
- Add 4.8 mL isopropanol and invert tubes to mix well. Centrifuge at top speed for 10 min, 10,000 RPM and 4°C. Pour out the supernatant and place each tube back in the rotor in the original orientation. Quickly centrifuge the tubes at maximum speed for several seconds. Aspirate all residual supernatant from the tubes carefully.
- Rinse the pellet with 1 mL ice-cold 70% ethanol, resuspending and centrifuging for 1 min to remove PEG. Discard all the residual supernatant.
- Air-dry and dissolve the plasmid DNA in 100 mL TE.

Step	Material	Purpose
1. Alkaline Denaturation	-	It breaks hydrogen bonds in DNA, denaturing the double helix.

2. Cell Growth and Harvesting	-	Grows colonies overnight in liquid broth; cells harvested by centrifugation.
3. Resuspension	Glucose, Tris, EDTA, Lysozyme	Maintains osmolarity, and pH, prevents nuclease activity, and may digest bacterial cell walls.
4. Lysis using Lysis Buffer	NaOH, SDS	Breaks cell membrane and denatures DNA.
5. NeutRalization	Potassium/Sodium Acetate	NeutRalizes the solution, allowing plasmids to renature while larger DNA remains denatured.
6. Ethanol Precipitation	Chilled Ethanol	Precipitates plasmid DNA, which can then be collected through centrifugation.
7. Resuspension	Water/TE Buffer	Plasmid DNA is resuspended in a suitable buffer for storage or further use.

Digestion and ligation

In the process of preparing the recombinant DNA construct, digestion and ligation are fundamental steps. These steps involve cutting the plasmid vector and the amplified gene of interest with specific restriction enzymes and then joining them together.

The two restriction enzymes used for digestion were EcoRI and NcoI. These enzymes recognize specific DNA sequences and cut the DNA at those sequences, generating cohesive ends or overhangs that are complementary to each other.

After isolating the eutR and eutS genes via PCR amplification, the next step was to insert them into the plasmid vector. This was achieved by digesting both the plasmid vector (in this case, the modified PQE60 plasmid containing the GFP gene) and the amplified genes with EcoRI and NcoI.

During digestion, EcoRI and NcoI recognition sites were strategically incorporated at the ends of the eutR and eutS genes through primer design. This ensured that the digested genes and the plasmid vector would have complementary overhangs compatible for ligation.

Host Preparation + Transformation

To prepare CaCl2-mediated E. coli DH5 Alpha competent cells, initially, a single colony of the DH5 Alpha strain was cultured in LA media. Subsequently, a primary culture was established by inoculating 1 colony into 10 ml of media, followed by a 1:100 dilution to generate a secondary culture, resulting in a total volume of 50 ml. The secondary culture was then incubated at 37°C and 180 RPM until reaching the early log phase, monitored by checking the optical density (OD) at OD600 intervals. Once the OD reached the desired range of 0.4 - 0.6, indicative of the early log phase, growth was arrested by chilling the sample on ice. After chilling, the sample was centrifuged at 4°C and 5000 RPM for 5 minutes to pellet the cells. The supernatant was discarded, and the cell pellet was resuspended in 4 ml of prechilled 0.1 M CaCl2 solution. Following an ice incubation of 15 minutes, the suspension was centrifuged again at 4°C and 5000 RPM for 5 minutes. The supernatant was once more discarded, and the cell pellet was resuspended in 400 µL of 0.1 M CaCl2 and 15% glycerol solution. Finally, aliquots containing 50 µL of the competent cells were prepared and stored at -80°C for future use.

The competent cells from the previous experiment were thawed on ice to initiate the transformation and recovery process of E. coli DH5 Alpha cells treated with CaCl2 solutions. Subsequently, isolated PQE60 plasmid DNA was added to the cells, and thorough mixing was achieved by pipetting the mixture up and down. The sample was then incubated on ice for 5 minutes to facilitate DNA uptake. Next, a heat shock treatment was administered to the competent cells by placing them at 42°C for 90 seconds in a dry bath, followed by immediate transfer to ice for a 2-3 minute incubation period. For recovery, pre-warmed LB media was added to the transformed cells and allowed to grow for 1 hour. The cells were then spun down, resuspended in approximately 100 µL of the medium, and plated onto LA media containing the ampicillin antibiotic. After an incubation period of approximately 18-20 hours, colonies were observed on the plates, indicating successful transformation. Specifically, the transformed cells with ampicillin antibiotic-resistant plasmid were identified among the colonies.

Screening

IPTG Protocol

We utilized pQE60 plasmid backbones pre-equipped with GFP (pQE60-GFP) for our study. GFP expression is controlled by the lac operon operator present in this plasmid. The lacI gene, encoding the repressor protein of the operon, is situated within the lac operon. When the repressor binds to the lactose metabolite allolactose, an allosteric change occurs, preventing the repressor from binding to the lac operator. Consequently, the lac operator becomes accessible for transcription in the presence of lactose, which is converted to allolactose by proteins within the lac operon.

In our plasmid design, the lac operator governs GFP expression. To confirm GFP expression from our plasmids, we utilized IPTG, an analog of allolactose, to de-repress the lac operator and induce GFP expression.

E. coli cells carrying pQE60-GFP plasmids were initially cultured overnight at 37℃ with shaking at 180 rpm in both tryptone broth (TB) and M9 media. Subsequently, cultures in both media were treated with IPTG to a final concentration of 500 µM, inducing His6-IPTG expression. After IPTG addition, a secondary culture was allowed to grow until reaching an OD of 0.4-0.6 for both media.

From the overnight culture, cells harboring pQE60-GFP plasmids were cultured differently, with various concentrations of IPTG. The final concentrations of IPTG used were: 0, 1 µM, 10 µM, 100 µM, and 1 mM for both M9 and TB cultures. To prepare these working concentrations, IPTG stock solutions were made as follows: 0, 100 µM, 1 mM, 10 mM, and 100 mM, respectively. Two replicates of each concentration were prepared.

The initial plate reading yielded no change, with no fluorescence detected. However, subsequent attempts proved successful, resulting in observable fluorescence. The successful result is detailed in the table below.

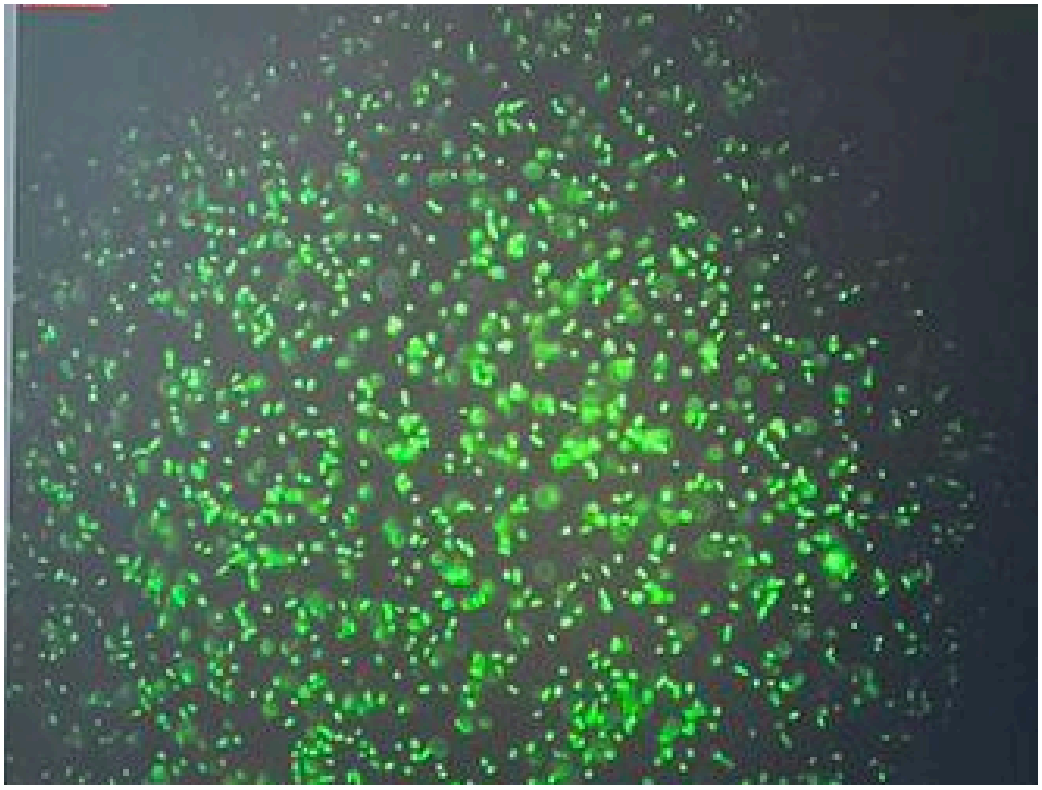
The cells were then observed under a fluorescence microscope, and the fluorescence intensity was quantified. We observed a fourfold increase in fluorescence in the tryptone broth cells compared to those in TB.

IPTG Results

Well no.	No_IPTG	IPTG	
	1	2	
A	943	4210	TB Media
B	467	876	
C	63	63	M9 Media
D	64	64	

Upon IPTG induction, a significant increase in fluorescence intensity is observed, indicating successful induction of GFP expression. Higher concentrations of IPTG result in higher fluorescence intensity, indicating a dose-dependent response. Moreover, a notable disparity in fluorescence intensity between TB and M9 media suggests that growth media composition strongly influences GFP expression levels, with TB media fostering substantially higher expression compared to M9 media.

Microscopy Observation of GFP



Sequence Conformation

Sequence Conformation refers to the process of verifying the genetic sequence of a target gene or DNA construct. In the context provided, if the Green Fluorescent Protein (GFP) expression hadn't been observed, indicating a potential failure in the biosensor development, it would prompt a closer examination of the DNA sequence.

The absence of GFP could suggest issues during the insertion of the vector into the host organism, such as mutations or errors in the genetic sequence.

Conclusion

In conclusion, the development of a biosensor for vitamin B12 in bacteria involved intricate genetic modifications, including the incorporation of eutS and eutR genes into a PQE60 plasmid. Despite initial PCR challenges, troubleshooting led to successful gene amplification. Subsequent steps, such as plasmid isolation, digestion, and transformation, enabled the integration of target genes into E. coli cells. Validation via GFP expression and microscopy confirmed biosensor functionality. This achievement underscores the power of genetic engineering and meticulous experimental design, promising diverse applications in healthcare and biotechnology.