Design and Application of γ-aminobutyric Acid Nano-Fluorescent Probe

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Abstract. The design and development of nano-fluorescent probes is a research hotspot in the field of biological functional materials. The construction of γ-aminobutyric acid (GABA) fluorescent probe based on fluorescence resonance energy transfer (FRET) technology will become a new tool for bacterial screening. Recombinant plasmids suitable for prokaryotic expression were constructed by subcloning technology, and verified by agarose gel electrophoresis and sequencing. The target protein was expressed in BL21 E. coli and purified by nickel matrix affinity chromatography. Spectroscopy and fluorescence imaging validate probe function and evaluate bacteria's environmental adaptability. The results showed that a recombinant plasmid suitable for efficient and stable expression of prokaryotes was successfully constructed. The release of GABA will cause changes in fluorescence intensity. The probe can evaluate the metabolic activity and adaptability of bacteria to different environments. This method uses bacteria's metabolic activity to realize the selection of bacteria. The sample does not require special biochemical treatment. Compared with traditional evaluation methods by counting the number of bacteria and analyzing metabolite, it is a more convenient tool for screening and evaluation of bacteria strains.

1. Introduction

Microbes are closely related to the human environment. Degradation, metabolism or enrichment by microorganisms can affect most organic compounds or inorganic metals [1]. Because of these unique properties, they are widely used in various industries, such as environmental protection [2], tertiary oil recovery [3], etc., which are inseparable from the role of microorganisms and their active molecules. The microbial genome is simple, and it is easy to carry out genetic modification through genetic engineering technology, which provides the possibility for establishing targeted engineering bacteria [4]. However, the application of various microbial technologies is still not widespread enough, mainly due to the complexity of microbial screening. At present, there are a large number of studies dedicated to the evaluation of highly efficient engineering bacteria, but they are more focused on the quantitative analysis of the number of bacteria [5], and lack evaluation tools for bacterial metabolic activity and environmental adaptability.

γ-aminobutyric acid (GABA) is a naturally active molecule that is widely found in animals, plants [6] and microorganisms, and has different physiological functions in different organisms. Related
research showed that GABA is involved in the metabolism of nitrogen and carbon sources in microorganisms, and putrescine and spermine [7, 8]. When the microorganism is subjected to environmental stimuli, it can respond to the corresponding pressure response. Under external environmental stress such as changes of pH, temperature [9], water content [10] and oxygen [11], the concentration of GABA in bacteria will change [12]. Therefore, the GABA concentration in bacteria reflects the metabolic activity and environmental adaptability of bacteria to a certain extent. Taking advantage of this feature of GABA, the construction of Fluorescence resonance energy transfer (FRET)-based GABA probes will provide new ideas for bacterial screening.

FRET technology developed on the basis of autofluorescent protein technology is a fluorescence detection technology for protein activity in living cells [13], which can monitor the conformational changes of proteins. This method induces the fluorescence resonance phenomenon of two fluorophores through the conformational change (binding/dissociation) of the protein. FRET can not only directly observe the dynamic spatial distribution of proteins in cells, but also detect whether protein activity has changed [14]. By designing GABA fluorescent probes, the concentration and distribution of GABA in living bacteria can be observed in real time, the changes in the metabolic activity of bacteria can be characterized, and the relationship between the metabolic activity of bacteria and the ability to adapt to the environment can be established, which is beneficial to the rapid screening of bacterial strains. GABA fluorescent probes have significant advantages in bacterial strain screening. First, the sampling volume is small. Second, samples can be directly detected without pretreatment, simplifying experimental steps and saving detection time. Finally, the biodegradability of the bacteria was more accurately evaluated from the aspect of bacterial activity.

2. Materials and Methods

2.1. Materials

2.1.1. Plasmids and Materials. The strains were DH5α and BL21 E. coli competent cells of Solarbio Company, and the prokaryotic expression vector plasmid was pRSETB.

2.1.2. Main Reagent. LB liquid medium, LB solid medium, ampicillin, T4 ligase, restriction enzymes, PCR kit, PCR purification kit, plasmid mini-extraction kit, gel recovery kit, isopropyl-β-D-thiogalactopyranoside (IPTG), imidazole, Trition X-100, metal nickel agarose affinity matrix, SDS-PAGE gel configuration kit, Coomassie blue protein gel rapid staining solution.

2.2. Methods

2.2.1. Design and Synthesis of PCR Primers. Based on the ECFP, YPet, and GABA<sub>B1</sub>-VFT gene sequences in Genbank, primers were designed using Oligo 7 software and the appropriate restriction sites and protective bases were introduced (table 1). The underlined lines indicate the protected bases, and the bolded lines indicate the restriction sites. In table 1, P1 and P2 are primers for ECFP amplification, P3 and P4 are primers for YPet amplification, and P5 and P6 are primers for GABA<sub>B1</sub>-VFT amplification. The primers were synthesized by Sangon Biotech.

| Number | Primers | Restriction sites | Sequences (5’ to 3’) |
|--------|---------|-------------------|---------------------|
| P1     | ECFP F  | BamH I           | CG/GGATCCG/ATGTTGAGCAAGGGCGAGG |
| P2     | ECFP R  | Xho I            | CG/CTCGAG/GGCAGCGGTCACTAGA |
| P3     | YPet F  | Kpn I            | CG/AGATCC/ATGTCTAAAAGTGAAAGAATTATCCA |
| P4     | YPet R  | EcoR I           | CCC/AAGGCTT/CATTTTGATACATTTTATCC |
| P5     | GB1 F   | Xho I            | CC/CTCGAG/ATGCGCCCAGGGGGG |
| P6     | GB1 R   | Kpn I            | CGG/GGTACC/GCCCAGGCTGGAGAGAAGACTG |
2.2.2. Subcloning. Using the GABA<sub>B1</sub> receptor in the existing plasmid and clone library containing ECFP and YPet as the PCR template, a large number of products can be obtained by PCR amplification with designed primers. The PCR amplification product was purified using a gel purification kit, digested with restriction enzymes as shown in figure 1, and the prokaryotic expression vector pRSETB was digested with BamHI and EcoRI, and the digested product was purified. T4 ligase was used to ligate the target gene and the vector plasmid recovered by digestion at 16 °C overnight and transduced into DH5α. The transformed bacterial was spread on LB solid medium containing ampicillin, screen for bacterial cells using ampicillin resistance, and finally use plasmid extraction kit to purify plasmid. The obtained recombinant plasmid was sent to Sangon Biotech for sanger sequencing.

![Figure 1. Schematic diagram of the recombinant plasmid structure.](image)

2.2.3. Protein Expression and Purification. Transducing the recombinant plasmid into BL21 E. coli competent cells. Pick the monoclonal colonies and add them to the LB liquid medium. When OD600 reached 0.6 to 0.8, IPTG inducer was added to a final concentration of 0.5 mM, cultured for another 4 h, the pellet was centrifuged. Using the Binding Buffer to resuspended the pellet, sonicated and centrifuged to collect the supernatant. Purify the protein of interest using nickel matrix affinity chromatography. Because the recombinant plasmid contains the His tag, the target protein can specifically bind to the nickel matrix. The target protein can be obtained by washing with a low concentration of imidazole and elution with a high concentration of imidazole. Finally, the protein dialysate and PBS were dialyzed to obtain the purified protein solution of interest. The size of the protein of interest was verified by SDS-PAGE electrophoresis.

2.2.4. Protein Function Verification. In order to verify the function of the probe, a full-wavelength microplate reader is used to perform a spectral scan in a wavelength range of 405 nm and an emission light of 450 nm to 600 nm, with a step size of 2 nm under the condition of dark; After the scan was completed, GABA was quickly added to the final concentration of 1 mM, gently shaken, and scanned again under the same spectral scanning conditions, and the fluorescence changes were compared to verify the function of the probe.

2.2.5. Evaluation of the Environmental Adaptability of Bacteria Using GABA Probes. The recombinant plasmid was transformed into BL21 E. coli competent cells, and the culture conditions of the bacteria were changed. Change the bacterial culture conditions, culture in acid, alkali, salt, low temperature and 37 °C. The changes of the bacteria’s fluorescence were observed, and the fluorescence intensity of the bacteria to obtain the adaptability of the bacteria environment was compared also.

3. Results

3.1. Recombinant Plasmid Construction

Agarose gel electrophoresis analysis of the amplified product obtained by PCR using the designed primers showed that in the plasmid construction scheme (figure 2), the length of ECFP is 684 bp, the length of YPet is 717 bp, and the number of genes of the ligand binding region of the GABA<sub>B1</sub>-VFT receptor is 1338 bp. The clear and bright specific bands can be seen from the electrophoresis results.
the size of which is the same as the theoretical value. The sequencing result of the obtained recombinant plasmid was completely consistent with the gene sequence of the plasmid construction scheme, and the recombinant plasmid was successfully constructed.

![Electrophoresis of PCR products](image1.png)

**Figure 2.** Electrophoresis of PCR products.

### 3.2. Protein Expression and Purification

Recombinant plasmid and unloaded plasmid were transformed into BL21 E. coli competent cells for expression, and the total proteins were detected by SDS-PAGE electrophoresis detection. The test results are shown in figure 3a. By comparing Marker and empty vector, the size of target band was consistent with the expected value in 112 kDa, which indicates the target protein is successfully expressed. The target protein was purified by nickel matrix affinity chromatography. The results of SDS-PAGE electrophoresis are shown in figure 3b.

![SDS-PAGE electrophoresis](image2.png)

**Figure 3.** SDS-PAGE electrophoresis.

### 3.3. Fluorescence Spectroscopy

To verify the function of the biosensor, the fluorescence emission spectra between 450-600 nm of purified target protein was measured upon excitation at 405 nm. A lower energy transmission efficiency was observed after adding a specific 1mM GABA (figure 4).

![Fluorescence spectrum](image3.png)

**Figure 4.** Fluorescence spectrum.
3.4. Fluorescence Imaging

The GABA probe was used to evaluate the adaptability to different bacterial environments. The recombinant plasmid was transduced into BL21 competent E. coli, and induced to culture for 4 hours under six different culture conditions. The photos were taken with FRET imaging equipment, as shown in figure 5. The fluorescence image under each condition is processed into a fluorescence value to obtain a histogram (n = 5) as shown in figure 6. It can be seen that the bacterial activity cultured under the optimal conditions is the best (37 °C, neutral Medium), followed by alkaline, salt, acidic conditions, low temperature (10 °C) and anaerobic conditions, the metabolic activity of bacteria is poor.

![Figure 5. FRET imaging of bacteria in different models: (a) Optimal culture conditions; (b) Low temperature culture conditions; (c) Alkaline culture conditions; (d) Acidic culture conditions; (e) Salty culture conditions; (f) Anaerobic culture conditions.](image)

![Figure 6. Fluorescence intensity under different models.](image)

4. Conclusion

Microbes are closely related to humans, and proper use can benefit humans. In the petroleum industry, the use of microorganisms for petroleum exploration, extraction, processing, and treatment of petroleum-contaminated soils and oceans has great development prospects. Rapid screening of the excellent engineering bacteria created by orientation can bring more benefits to human production and life.

As an important bacterial stress response factor, GABA participates in multiple metabolic pathways of bacteria and is closely related to the metabolic activity and environmental adaptability of bacteria (figure 7). The use of FRET technology to design biological probes for real-time monitoring of GABA in bacteria is of great significance for the evaluation of bacterial environmental adaptability. Based on
the analysis of the database and software, a recombinant plasmid construction scheme was established in this paper. Recombinant plasmids suitable for expression in prokaryotes were successfully prepared by using subcloning technology. BL21 E. coli competent cells successfully expressed the target protein, and the purified target protein solution was obtained by nickel matrix affinity chromatography. The function of the probe was verified by fluorescence spectrum. Finally, probes were used to evaluate the adaptability of bacteria in six growth models.

Figure 7. Metabolic pathways of GABA in bacteria under environmental stress.

It can be seen through the bacterial metabolism in the model that the production environment has a significant effect on the growth and metabolism of microorganisms. Due to the significant differences in the oilfield environment in different regions, the selection of targeted and efficient bacterial strains has become the key to the promotion and application of microbial oil recovery technologies. The traditional screening engineering is complicated. Screening the most suitable reservoir engineering bacteria from many strains is often time-consuming, labor-intensive, costly, and ineffective. The successful construction of GABA probes based on FRET technology provides new ideas for rapid screening of petroleum engineering bacteria, research and development and development of new strains with a wider range of adaptation. By simply observing the fluorescence intensity, we can achieve rapid screening of a variety of bacteria, which greatly simplifies the steps of bacteria screening. And it has breakthroughly characterized the environmental adaptability of microorganisms in terms of bacterial metabolic activity, making it more efficient and reliable. In the future, we will use this probe for further experiments. By transforming different reservoir engineering bacteria and observing the change of fluorescence, we can quickly screen new reservoir engineering bacteria with high efficiency and wide application range.

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