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# Visualized and precise design of artificial small RNAs for regulating T7 RNA polymerase and enhancing recombinant protein folding in *Escherichia coli*

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Small non-coding RNAs (sRNAs) have received much attention in recent years due to their unique biological properties, which can efficiently and specifically tune target gene expressions in bacteria. Inspired by natural sRNAs, recent works have proposed the use of artificial sRNAs (asRNAs) as genetic tools to regulate desired gene that has been applied in several fields, such as metabolic engineering and bacterial physiology studies. However, the rational design of asRNAs is still a challenge. In this study, we proposed structure and length as two criteria to implement rational visualized and precise design of asRNAs. T7 expression system was one of the most useful recombinant protein expression systems. However, it was deeply limited by the formation of inclusion body. To settle this problem, we designed a series of asRNAs to inhibit the T7 RNA polymerase (Gene1) expression to balance the rate between transcription and folding of recombinant protein. Based on the heterologous expression of *Aspergillus oryzae* Li-3 glucuronidase in *E. coli*, the asRNA-antigene1-17bp can effectively decrease the inclusion body and increase the enzyme activity by 169.9%.


## 1. Introduction

Recently, many sRNAs have been identified and characterized in diverse organisms, particularly in *Escherichia coli* [1,2]. They have been divided into cis-acting sRNA (csRNAs) and trans-acting sRNAs (tsRNAs) [3]. The tsRNAs usually interact with the 5′ un-translated region (5′UTR) or the translation initiation region (TIR) of target mRNA by base pairing to regulate gene expression [4,5]. The tsRNAs have been used as versatile and flexible genetic tools in many fields, such as building logic gate in synthetic biology [6], knocking down shunt pathway in metabolic engineering [7] and studying bacterial physiology [8]. Since they are such a powerful genetic tool that many groups have tried to develop asRNA to regulate gene expression.

Yokobayashi et al. [9] developed an efficient throughout screening method for asRNAs by designing antisense domain as a series of N bases, and fusing randomized antisense domain with natural sRNA scaffolds. However, the operation was complicated and time consuming. To simplify the operation, Lee [10] rationally designed the antisense domain of asRNA by introducing complementary base pairing with the front of target 24bp sequence. This can deeply predigest the screening process, but the inhibition efficiency of designed asRNAs was dramatically different. Apart from the antisense domain, Lee [11] and Ikebukuro [12] found that the replacement of sRNA scaffolds could also improve the efficiency of asRNAs. But sRNA scaffolds still need to screen efficient antisense domain to gain effect.

Based on previous study, the key step to implement reasonable design of asRNAs was to design efficient antisense domain, so we focus on designing efficient antisense domain.

T7 expression system was one of the most popular protein expression systems [13] in which T7 RNA polymerase specifically recognized T7 promoter [14] and elongated target mRNA 5-fold faster than *Escherichia coli* RNA polymerases [15]. It includes two parts: a plasmid containing T7 promoter and a factitial DE3 lysogenic host [16]. The DE3 lysogenic host has one copy of T7 RNA polymerase gene (Gene1), which was pre-integrated into genome to produce a very limited amount of T7 RNA Polymerase [17]. The

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pET expression system from Novagen Inc. was one representative of such approach and has been widely used for protein expression in *E. coli* [18]. Although the amount of T7 RNA polymerase was very limited, the speed of target gene transcription was still too fast. The high-level expression of recombinant proteins in *Escherichia coli* often accumulate as insoluble aggregate in vivo as the inclusion body [19] that severely limit its application.

We proposed that the inclusion body formation was due to the high efficiency of T7 RNA polymerase. The inclusion body is very likely to form due to the imbalance between the speed of target gene transcription and protein folding. In this study, we developed a series of asRNAs with different inhibition efficiency to fine turn Gene1 expression, which dramatically decreased the inclusion body formation of T7 expression system.

## 2. Materials and methods

### 2.1. Strains, plasmids and culture condition

*Escherichia coli* Trans1-T1, TOP10 were selected as the clone hosts and BL21 (DE3) as the expression host. Plasmids pEASY-Blunt (TRANSGEN BIOTECH), pSB1A3, pSB1C3 and pSB1K3 (parts from iGEM) were used to express asRNAs. To verify the function of asRNAs in optimizing the T7 expression system, plasmid pET-28a-pGUS which expressed *Aspergillus oryzae* strain Li-3 glucuronidase (GenBank: EU095019.1) was used as a model system. Since the recombinant *Aspergillus oryzae* strain Li-3 glucuronidase (pGUS) was expressed as the inclusion body that severely limit our study. The strains were cultured in Luria-Bertani (LB) media at 37 °C, 30 °C and 16 °C with 200 rpm. Restriction enzymes and other modifying enzymes were purchased from Thermo Fisher Scientific and used according to the manufacturer's recommendations.

### 2.2. Plasmids and strains construction

Plasmid pSB1A3-system was constructed as a platform to express asRNA and to test its inhibition efficiency. This vector contains both asRNA expression cassette and verification cassette that was developed by BioBrick method [20] (Fig. 1). Besides, pSB1C3-asRNA cassette was constructed to express asRNAs. And the pSB1K3-verification casette was constructed to verify the efficiency of asRNAs.

By one step mutation, 24bp of ipgC, sicA* and esxC 5'leader mRNA sequence were fused to the reporter gene *LacZ* to get pSB1A3-system-ipgC, pSB1A3-system-sicA* and pSB1A3-system-esxC. The complementary base pairing 24bp sequence of ipgC, sicA* and esxC 5'leader mRNA were fused to sRNA scaffold MicC in the same way to get pSB1A3-system-ipgC-anti-ipgC-24bp, pSB1A3-system-sicA*-anti-sicA*-24bp and pSB1A3-system-exsC-anti-exsC-24bp.

Besides, pSB1A3-system-ipgC-anti-ipgC-20bp and pSB1A3-system-exsC-anti-exsC-20bp have been constructed to verify whether the exposure of the antisense domain is necessary for asRNA to show function. Furthermore, pSB1A3-system-ipgC-anti-ipgC-15bp, pSB1A3-system-ipgC-anti-ipgC-10bp and pSB1A3-system-ipgC-anti-ipgC-5bp were developed to test the efficiency of the length of antisense domain.

To optimize the T7 expression system, asRNA-anti-gene1-17bp, asRNA-anti-gene1-13bp and sRNA-anti-gene1-10bp were designed to inhibit Gene1 expression independently to see whether these asRNAs can decrease the inclusion body of pGUS and improve its activity.

These asRNAs were developed by fusing 17bp, 13bp and 10bp with complementary base pairing sequence of Gene1 5'mRNA respectively to MicC scaffold. Using plasmid pSB1C3-sRNA cassette as template the plasmids pSB1C3-asRNA cassette-anti-gene1-17bp, pSB1C3-asRNA cassette-anti-gene1-13bp and pSB1C3-asRNA cassette-anti-gene1-10bp were constructed. After amplified by primer pair Primer SphI-fw & Primer BglII-rv, the PCR fragments were digested with SphI and BglII, gel-purified, and then ligated into plasmid pET-pGUSE, which was digested with the same enzyme pair. The final plasmids were named pET-28a-pGUSE-asRNA-anti-gene1-17bp, pET-28a-pGUSE-asRNA-anti-gene1-13bp and pET-28a-pGUSE-asRNA-anti-gene1-10bp respectively.

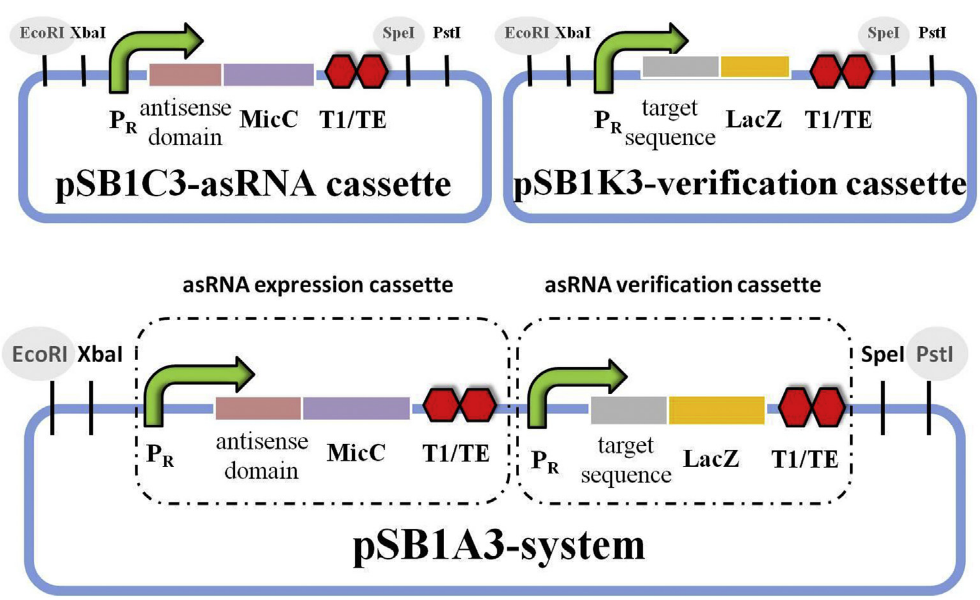


**Fig. 1.** The schematic of the design and construction of asRNA expression and verification system. The plasmid pSB1C3-asRNA cassette was consisted of promoter $P_R$, antisense domain of the asRNA which was reversely complement base pairing with the target inhibition sequence, MicC scaffold of asRNA and the bi-directional terminator T1/TE. The plasmid pSB1K3-verification cassette was consisted of promoter $P_R$, initial 24bp sequence from the target inhibition gene to fuse with the reporter gene LacZ which has deleted the ATG. The plasmid pSB1A3 was consisted of both asRNA expression cassette and asRNA verification cassette. It was constructed by BioBrick method.