ENGINEERING A PLASMID AS A REPORTER SYSTEM
FOR QUANTIFYING GENE EXPRESSION
IN *ESCHERICHIA COLI*

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Abstract

Different gene reporter assays are used for quantifying various methods for synthetic control of gene expression. The activity of a reporter protein, fluorescence or chemical stain, is usually proportional to the level of mRNA produced in the cell and can be utilized for monitoring the activity of a promoter or enhancer. Such a reporter gene is the lacZ from *Escherichia coli*, encoding β -galactosidase. We cloned the PL promoter from phage lambda into the pRS414 plasmid of over 10kb to create a gene reporter assay. A KpnI restriction site was introduced into the plasmid used for inserting the promoter together with a ribosomal binding site for bacterial and archaeal mRNA and the initiation of transcription codon ATG in frame with the lacZ. B-galactosidase activity assays showed a correlation between the induced expression of the lacZ gene and the time, indicating the rightness of the construct, which can be used for lacZ reporter gene activity assays and synthetic control of gene expression in synthetic biology studies. The new plasmid named pRS414ge is very suitable for RNA synthetic biology experiments.

Key words: DNA cloning, restriction site, promoter, LacZ gene, β -galactosidase assay, RNA synthetic biology

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1. Introduction. Bacterial operons are polycistronic transcripts that can produce multiple proteins from one mRNA transcript [1]. The lactose operon (lac operon) is required for lactose transport and metabolism in *Escherichia coli* (*E. coli*) [2]. LacZ gene translates for the β -galactosidase, an intracellular enzyme that cleaves lactose, a disaccharide, into glucose and galactose as carbon energy sources [3]. A colour indicator for β -galactosidase activity is the Ortho-nitrophenyl- β -D-galactopyranoside (ONPG), which is cleaved to produce an intensely yellow compound, the orthonitrophenol. This compound absorbs a 420 nm wavelength and enables the measurement of the β -galactosidase activity [4]. β -galactosidase hydrolyzes the o-nitrophenyl- β -D-galactopyranoside substrate that yields stoichiometric amounts of yellow o-nitrophenol and colourless galactose. The lacZ has been used as a reporter gene in gene-expression systems not only in *E. coli* but also in several microbial species [5–8]. The usage of the lacZ gene together with the initiation codon ATG and the *E. coli* consensus Shine–Dalgarno site has been used from early studies for the generation of lacZ reporter systems for monitoring transcription in operon fusion [9]. The presented work used the pRS414 plasmid of about 10kb to create a new lacZ reporter system comprising a promoter, the Shine–Dalgarno site, and the ATG codon in the frame of the lacZ gene. The promoter element inserted was the 38 bp sequence of the λ phage PL constitute and strong promoter [10]. The β -galactosidase assay revealed high activity levels in the samples compared to the negative control lacking the λ phage promoter that showed no activity. The monitoring of β -galactosidase can also be used as a marker for examining the effects of the activity of allosteric ribozymes and oligonucleotides. The newly generated system can be applied in synthetic biology applications for the artificial control of gene expression for designer ribozymes and antisense oligonucleotides [11–13], or other gene control elements can act as switches for control of gene expression.

2. Materials and methods. 2.1. Generation of the lacZ reporter system. A DNA fragment bearing a KpnI restriction site, the Shine–Dalgarno sequence, and the initiation of transcription codon ATG in frame with the lacZ was created via hybridization with two deoxyoligomers (Fig. 1). The first fragment introduced the KpnI site in the pRS414 plasmid utilizes BamHI and XhoI restriction sites located on the plasmid (Fig. 1A). The cloning used the competent *E. coli* bacteria of the HB101 strain (Takara Bio INC, Japan). The bacteria were grown in LB media containing ampicillin (100 mg/L). The introduction of the KpnI site was verified via restriction mapping of the generated clones by KpnI endonuclease (Fig. 1B). A 1% agarose gel stained with GelRed (Biotium, Fremont, CA) was used to visualize the restriction mapping. The PL promoter sequence of 38 bp was cloned between the BamHI and the new KpnI restriction site (Fig. 1C). The promoter fragment was generated via the annealing of the deoxyoligomers (Fig. 1). The rightness of the cloned sequences was verified by sequencing (Macrogen Europe, Netherlands). The pRS414ge map is de-

Fig. 1. A. Introduction of the DNA sequence in the plasmid. The fragment composed of the KpnI, SD, and ATG codon in the frame to the lacZ was inserted in the BamHI and XhoI restriction sites present in the plasmid polylinker. B. Restriction mapping verifying the presence of the KpnI site after the insertion. Lane 1 shows the digested control plasmid without the inserted DNA fragment bearing the KpnI site. Lanes 2 and 3 show the digestion of two plasmids with KpnI after introducing the DNA fragment bearing the KpnI site. Both plasmids were linearized. C. Cloning strategy of the pL promoter sequence of *E. coli* in the plasmid. The BamHI and KpnI sites were utilized to insert the promoter fragment. D. Enzymatic activity of β -galactosidase. Absorptions were recorded at 15, 20, and 30 min. The Miller Units (MU) are calculated according to the equation [14]. The last line is the recorded activity of the negative control, the plasmid without the insertion of the promoter fragment at the 30 min time point. E. The completed sequence. The sequence of all the inserted components in the plasmid's polylinker after the cloning procedure

picted in Fig. 1E. The complete sequence of the pRS414ge plasmid is given at <https://penchovsky.atwebpages.com/research.php?page=11>.

2.2. *B-galactosidase* assay. A β -galactosidase assay was performed to quantify the level of promoter activation. *E. coli* cells were grown overnight in LB medium containing ampicillin (100 mg/L) and incubated at 37°C with shaking. A permeabilization solution of 80 μ l of (100 mM dibasic sodium phosphate (Na_2HPO_4), 20 mM KCl, 2 mM MgSO_4 , 0.8 mg/mL CTAB (hexade-

cyltrimethylammonium bromide), 0.4 mg/mL sodium deoxycholate, 5.4 μ L/mL beta-mercaptoethanol) was added, after which the absorption at 600 nm was measured (Abs 600). The liquid culture of 20 μ L was added to tubes containing the permeabilization solution and 600 μ L of substrate solution containing ONPG, and the time of the addition was recorded. From each sample, three repetitions were made for three-time points of 15, 20, and 30 min, respectively. After sufficient colour had been developed, 700 μ L of stop solution (1 M Sodium Carbonate (Na_2CO_3)) was added and mixed well. Next, the tubes were transferred to a microfuge and centrifuged at full speed for 5–10 min. The supernatant was removed into cuvettes, and the absorption was measured at 420 nm (Abs 420). Miller formula [14] was used to calculate Miller Units (MU) of enzyme activity.

$$\text{MU} = 1000 \times \frac{\text{Abs 420}}{\text{Abs 600} \times V \times T}, \quad [14]$$

where T is the time of reaction in minutes and V is the volume of culture used in the assay. Each experiment is triplicated, and the average of their A420 was used to calculate the MU for each sample.

3. Results. 3.1. *B-galactosidase activity assay.* High levels of enzymatic activity were recorded in all β -galactosidase assays throughout the three tested time points of 10, 20, and 30 min, respectively. For the first time point of 10 min, 774.56 MU was obtained. Similar activity was obtained for the other time points with 773.99 MU and 763.48 MU of the 20 and 30 min, respectively. The strong constative λ phage promoter expressing the lacZ gene gave similar results for all three-time assay points. All three-time points of the assay with the PL promoter have shown MU above 760, indicating a higher level of enzymatic activity. The negative control sample lacking the λ phage promoter did not show any enzymatic of the β -galactosidase at the 30 min time point as expected due to the lack of the champion from the plasmid (Fig. 1D). In the absence of the promoter, the β -galactosidase assays showed 11.08 MU only as a background signal. Those results indicate that the generated gene reporter construct utilizing the pRS414 plasmid worked efficiently and delivered the expected results about the enzymatic activity of the β -galactosidase amongst the tested sample and the negative control lacking the PL promoter at times points that were tested.

4. Discussion. We have demonstrated that the new reporter lacZ system for RNA synthetic biology studies made a new pRS414ge gene reporter plasmid. For the creation of the reporter system, a DNA fragment bearing a KpnI site, the Shine–Dalgarno site, and the initiation of transcription ATG codon in the frame to the lacZ gene, we have added in the plasmid's BamHI and XhoI restriction sites. The presented research introduced the λ phage PL promoter sequence of 38 bp in the plasmid as a BamHI – KpnI fragment, using the new KpnI site introduced at the previous step. The β -galactosidase activity assay confirmed the rightness of the expression system as enzymatic activity was obtained in all

Fig. 2. The map of the pRS414ge plasmid (10715 bp). The map of the generated plasmid, including the KpnI site inserted restriction and the PL promoter

samples tested except the negative control. The λ phage PL strong promoter expressed the reporter lacZ gene constitutive all time, providing similar results during the activity assay.

The newly generated system has been successfully used for synthetic control of gene expression for designer ribozymes and antisense oligonucleotide, which will be published elsewhere. The plasmid utilized for the project is not the new pRS414 plasmid of 4788bp and has been designed to apply the synthetic expression of genes in *E. coli*. The complete sequence of our new pRS414ge plasmid containing the PL promoter (Fig. 2) of the λ phage is given and has a length of 10715 bp. The new pRS414ge is a unique tool for gene control experiments in RNA synthetic biology because it offers one-step cloning of different allosteric ribozymes, Shine–Dalgarno sites, and target sites for antisense oligonucleotides together with a very convenient and accurate gene expression array. The presented plasmid is available for academic researches after request.

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