A genetic toolkit for co-expression of multiple proteins of diverse physiological implication

Ali Samy Abdelaal a,c, Syed Shams Yazdani a,b,*

a Microbial Engineering Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India
b DBT-ICGEB Centre for Advanced Bioenergy Research, International Centre for Genetic Engineering and Biotechnology, New Delhi, India
c Department of Genetics, Faculty of Agriculture, Damietta University, Damietta, Egypt

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ABSTRACT

Construction of plasmids is crucial for expression of functional proteins of diverse physiological impact in E. coli. Here, we first designed and constructed a novel pair of bacterial expression vectors, i.e., pAS01 and pAS02, to be co-transformed with pQE30 for the co-expression of three target genes. The three plasmids contain ColE1, p15A and pSC101 origin of replication for high, medium and low copy plasmids, respectively, and same promoter (T5) and RBS. We then cloned genes encoding three reporter proteins (GFPuv, TurboRFP, and EYFP) in each of these plasmids and co-expressed in E. coli in six different combinations. Each of these reporter proteins exhibited diverse impact on growth, plasmid copy number and stability, and expression of other co-expressed proteins. Our results indicate that GFP and RFP were the most and the least favorable proteins for the cells, respectively, in terms of these parameters, especially on impacting expression of other co-expressed proteins.

1. Introduction

Nearly 50 years have passed since the first production of recombinant DNA molecules [1] and construction of bacterial plasmids in vitro [2]. However, there is still a shortage of manufacturing capacity due to the increasing demand of diverse recombinant proteins, which requires a much better approach in the optimization of production strategies and expression vector design. Efficient strategies for the development of recombinant proteins are becoming particularly important as more applications that demand significant amounts of high-quality proteins enter the industry. Moreover, expressing multiple proteins of diverse nature is the major thrust area to fulfill the goal of metabolic engineering and synthetic biology. Plasmids are extrachromosomal highly folded circular self-replicating cytoplasmic DNA elements present in most bacteria. Since the advent of genetic engineering, they have been used as molecular vehicles for recombinant genes. Plasmid-based expression is the most common option when expressing genes in prokaryotes [3].

Plasmids are commonly found in variable copy numbers in bacteria and they inflect a metabolic burden on the host cells, as they utilize the cellular resources for their replication and recombinant protein production. This metabolic burden correlates with plasmid copy number and toxicity of the inducers toward the host, which influence the bacterial growth rate as well as its products [4]. A common observation is that low- and medium-copy plasmids provide much more benefits over high-copy plasmids, such as tighter control of gene expression, the ability to replicate larger genes, and lower metabolic burden on the host cells [5]. Additionally, use of high-copy plasmid to enhance gene expression may rather lead to limitation in gene expression due to plasmid metabolic burden. Moreover, protein expression at very high levels might be harmful and, in this case, low- or medium-copy plasmids may be sufficient or even desirable [6]. For the production of peri-plasmatically secreted proteins, it was found out that the expression rate should be optimized rather than maximized [7].

There are many factors related to the expression vector that influence the recombinant protein production in E. coli. For example, adequate origin of replication for the desired plasmid copy number, regulation of transcription for adequate protein expression, promoter with an ease of induction, and translational features at the 5′-end of the mRNA transcript including ribose binding site (RBS) and the sequence between the RBS sequence and the start codon [4, 8, 9].

Reporter genes play a key role in gene expression studies. They are most commonly used as indicators of translational activity within cells. The cells are assayed for presence of the reporter by directly measuring the amount of the reporter protein. Fluorescent proteins are considered...
as ideal reporters which do not require substrates [10]. The green fluorescent protein (GFPuv) has a single excitation peak centered at about 395 nm, with an emission peak at 509 nm, while turbo red fluorescent protein (TurboRFP) has its excitation peak at around 553 nm, and an emission peak near 527 nm. These three fluorescent proteins are very commonly used in the laboratory studies for various applications, however, a relative efficiency of their expression and co-expression have not yet been performed.

Various approaches have been employed for the co-expression of multiple proteins in bacteria, especially when performing metabolic or pathway engineering. These include the use of operons, dual promoter systems, bidirectional promoters (BDPs), or co-transformation of more than one vector [11–14]. Each of these is associated with a number of limitations such as uneven or unreliable protein expression levels, silencing of some promoters, increased toxicity to cells when using multiple inducers, or availability/generalizability of BDPs.

The main objective of our study was to understand the behavior of multiple proteins of different physiological implications on the bacterial silencing of some promoters, increased toxicity to cells when using more than one vector systems, bidirectional promoters (BDPs), or co-transformation of more than one vector [11–14]. Each of these is associated with a number of limitations such as uneven or unreliable protein expression levels, silencing of some promoters, increased toxicity to cells when using multiple inducers, or availability/generalizability of BDPs. To achieve this objective, we created a new vector system that can accept three genes/operons to be co-expressed at different expression levels for various studies. This system consists of three plasmids, each carrying the three genes/operons to be co-expressed at different expression levels for the toolkit, co-expression of the different fluorescent proteins have been demonstrated at various expression levels.

2. Materials and methods

2.1. Strain and plasmid construction

Table 1. Plasmid construction and DNA manipulations were performed using standard molecular cloning protocols. Plasmid maintenance and propagation were performed in E. coli DH5α strain.

The vector pQE30 was purchased from Qiagen, while pZa31MCs and pZs21MCs were purchased from Expressys and used as the backbone plasmids. To create pAS01 and pAS02 plasmids, the DNA sequence containing Ptaeto-1 promoter with RBS and partial MCS from pZa31MCs and pZs21MCs plasmids were replaced with the DNA sequence containing T5 promoter, RBS, His-tag, and MCS from pQE30 using AatII and HindIII restriction enzymes. The construction of pAS01 and pAS02 has been described in detail in Fig. 1 and their sequences have been deposited to NCBI repository with GenBank accession numbers OL542782 and OL542783, respectively.

2.2. Cloning and transformation of genes encoding fluorescent protein

Genes encoding traffic light fluorescent proteins GFPuv, TurboRFP, and EYFP were amplified from their corresponding vectors pSs9, pTurboRFP-N, and pCH47742:pFCFP:Cas9YFP vector, respectively, and each gene was separately cloned into pQE30, pAS01, and pAS02 vectors to generate all possible nine combinations. All expressed proteins had 6-histidine tag at their N-terminus for detection using Western blot.

The pGFPuv coding sequence was amplified from the pSs9 vector as template using GFPuv-F/GFPuv-R primers and inserted into the pQE30, pAS01, and pAS02 vectors to generate all possible nine combinations. All expressed proteins had 6-histidine tag at their N-terminus for detection using Western blot.

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2.3. Sample preparation

Samples were prepared for all the analysis as follows. The bacterial strains were grown in 5 ml LB medium (pH ~7) with the appropriate antibiotics at 37 °C and 180 rpm in an incubator shaker (Eppendorf) and were induced using 0.5 mM IPTG at OD600= 0.6. Samples were collected after 4 h of induction for all analysis, except for real-time PCR where samples were collected after 2 h of induction.

2.4. Quantitative measurement of plasmid copy number

The plasmid copy number for each expressing clone was determined using real-time PCR as described earlier [15]. The sample for real-time PCR was prepared using the iQ™ SYBR Green Supermix (Bio-Rad)
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following the manufacturer’s instructions and the PCR was performed on the iCycler Thermal Cycler (Bio-Rad) using the SYBR-490 filter for fluorescence detection. The experiment was performed in biological triplicate for analysis of plasmid copy number.

Plasmid copy number relative to the chromosome was calculated using the following equation: Plasmid copy number = $2^{(C_{t \text{ chromosome}} - C_{t \text{ plasmid}})}$.

2.5. Standard plate count method

To obtain the population of cells bearing each of the plasmid, colony-forming units (CFU) were obtained against each antibiotic as follows. A volume of culture was aliquoted from each sample when $OD_{600}$ reached 0.4 and serial $10^5$-fold dilutions were made in LB medium and spread on 4 LB-agar plates, 3 plates having 3 different antibiotics, i.e., ampicillin, chloramphenicol and kanamycin, and 1 control plate having no antibiotic. Colonies were counted after 14 h of incubation at 37°C.

2.6. Gene expression analysis

Gene expression analysis was done by real-time PCR as follows. Total RNA was isolated from each sample and the expression levels of transcripts of all fluorescent genes in different strains were determined using primers listed in Table 1. rrsA (16S ribosomal RNA) gene was used for normalizing RNA expression.

Recombinant protein production was analyzed using Western blot. Total protein was isolated from each sample and separated using SDS-PAGE. Proteins were then transferred to nitrocellulose membrane, followed by immunodetection of proteins using monoclonal anti-poly histidine–peroxidase antibody from Merck.

2.7. Microscopy and image generation

Samples were pelleted and properly resuspended in 1X Phosphate buffer saline (PBS), placed on glass slide under coverslip, and scanned under microscope. A series of images were digitized with a confocal laser scanning microscope (A1r from Nikon) using different filters. The images were analyzed using Nikon NIS-Elements Advanced Research.

2.8. Data analysis

Data were analyzed in Microsoft Excel program and are presented as mean and standard deviations (SD) based on triplicates from at least three independent experiments.

Two-way analysis of variance (ANOVA) was used to compare the effects of strains and fluorescent proteins in qPCR and FACS. The least significant difference (LSD) values were used to compare means ($p \leq 0.05$ and 0.01). The Pearson Correlation was used to measure the strength and direction of linear relationships between pairs of continuous variables. These data were analyzed using IBM SPSS Statistics, version 20.

2.9. Fluorescence-activated cell sorting (FACS) analysis

For FACS study, cells were harvested 4 h post-induction, pelleted and resuspended in PBS. The cells were then analyzed on a BD FACSAria III for quantitative assessment of GFPuv (405 nm laser for excitation, 450/40 filter for detection), TurboRFP (561 nm laser for excitation, 610/20 filter for detection), and EYFP (488 nm laser for excitation, 530/30 filter for detection) fluorescent-producing cell population using an appropriate compensation matrix. Final data, given as log CFU/mL, resulted from at least three independent experiments with three replicates each. Data were analyzed using FlowJo software.

3. Results and discussion

3.1. Design and features of pAS01 and pAS02 vectors

For obtaining high yield of a recombinant protein, often high-level expression of the gene is needed using high copy number plasmid. But it has also been observed that high copy plasmid is counterproductive for some proteins. Further, in metabolic engineering, some enzymes need to be produced at lower level for optimizing the stoichiometry of a pathway. In general, the host cells suffer from high expression of recombinant proteins since it drains out cellular energy, thereby disrupting essential host cell functions. Besides, the protein of interest can be toxic to the cell. Our toolkit has been designed for use in metabolic engineering experiment for stoichiometry optimization of expression and co-expression of multiple proteins.

The criteria for an effective E. coli co-expression vector include the existence of a strong promoter, a compatible origin of replication, a rich multiple cloning site, and a suitable antibiotic resistance. In order to achieve this goal, we constructed two vectors, pAS01 with chloramphenicol resistance and pAS02 with kanamycin resistance, to be used for co-expression with commercial vectors bearing either ampicillin or any different resistance marker.

The new vectors were constructed in the following manner. The pAS01 was obtained by combining portion of the pZA31MCS vector and...
the pQE30 vector together. The resultant vector, pAS01 (2135 bp), carries p15A replication origin and the chloramphenicol resistance gene (Cmr) of pZAS1MCS and the T5 promoter, synthetic ribosomal binding site RBSII and 6xHis-tag coding sequence of pQE30 (Fig. 1). pAS02 vector was constructed by ligating portion of the vector pZAS21MCS and the vector pQE30 together. The recombinant vector, pAS02 (3709 bp), carries pSC101 replication origin and the kanamycin resistance gene (KanR) of pZAS21MCS and T5 promoter, synthetic ribosomal binding site RBSII and 6xHis-tag coding sequence of pQE30 (Fig. 1).

The three vectors used in our further study, i.e., pQE30, pAS01, and pAS02, had the ability to co-exist with each other due to different origin of replications. At the same time, all the vectors contained same promoter and RBS to ensure that differences in the expression of the cloned genes is only due to differences in plasmid copy number and not due to differences in the promoter or RBS strength. This technique also avoids use of multiple inducers that might have adverse impact on the growth and physiology of the host.

### 3.2. Generation of recombinant plasmids for expression and co-expression of traffic light fluorescent reporter proteins

To study the usefulness of the modified plasmids and the toolkit, we considered genes encoding GFPuv, EYFP, and TurboRFP proteins that emit green, yellow and red fluorescence, respectively, and have minimal overlap in their excitation/emission wavelengths. The three genes were separately cloned into each of the three plasmids under study to get nine different constructs in order to express each gene in different expression vector with different expression level (Fig. 2). All constructs were confirmed using PCR and DNA sequence analysis. These nine constructs were subsequently employed for co-transformation in different

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**Fig. 2.** Cloning of genes for fluorescent proteins GFPuv, TurboRFP and EYFP in three different plasmids, pQE30, pASA01 and pASA02.
3.3. Impact of gene type on plasmid copy number

Plasmid copy numbers are likely to vary when genes coding for different kind of proteins are cloned and expressed. Using three traffic light reporter genes as a basis to determine the variation in the plasmid copy number, we measured the plasmid copy number using real-time qPCR method when the cells were at their early induction phase, i.e., 2 hrs post-induction. qPCR amplifications of the genes from nine strains of *E. coli* were performed using GFPuv, TurboRFP, and EYFP as plasmid genes, and rrsA as chromosomal gene. Relative quantification between each plasmid gene and chromosomal gene were calculated, as shown in Fig. 3A. As expected, the copy number of pQE30-based plasmid was highest in the cases where GFPuv and EYFP genes were cloned. However, the same was not true for TurboRFP gene where the copy number pQE30-based plasmid was much lower, close to the pAS01-based plasmid. Based on the type of the gene cloned, it appears that cells decided to control the plasmid copy number, with highest for GFPuv (17 copies), followed by 10 copies for YFP, and lowest (2.8 copies) for RFP. The medium copy pAS01-based plasmid remained at 2–3 copies for GFP and RFP, while it reduced to <1 copy for YFP. The low copy pAS02-based plasmid was maximum for GFP (3 copies), while it reduced to 1 copy for RFP and <1 copy for YFP. It became evident from these results that the concept of high, medium and low copy numbers of plasmid based on the types of origin of replications is not absolute and can change depending upon the kind of heterologous genes that it carries. It is also known from the previous studies that plasmid copy remains significantly lower in logarithmic phase of growth as compared to the stationary phase [16, 17], reflecting overall lower plasmid copy number in our study.

3.4. Profiling of gene expression upon replication origin swapping

Differences in the copy number of the plasmids because of their unique origin of replication will have implications to the expression of the gene [18]. One would like to believe that increasing the copy number of a plasmid would lead to increased gene dosage [4, 19]. We have seen in the previous section that plasmid copy number may not be uniform for all the genes. Here, we tested how the expression of the three traffic light reporter genes varied with the change in origin of replication.

The expression levels of the target genes (GFPuv, TurboRFP, and EYFP) were normalized with respect to that obtained using pQE30. When the expression levels of the GFPuv gene in the three constructs were compared, it was found that pAS01-based and pAS02-based plasmid expressed 10-fold less and 20-fold less GFP transcript as compared to pQE30-based plasmid (Fig. 3B). This kind of transcript profile was expected as per the origin of replication these plasmids were bearing. However, same was not true for RFP expression where the pAS01-based and pAS02-based plasmid exhibited ~4-fold higher expression than pQE30-based plasmid (Fig. 3B). This clearly indicated that high copy number plasmid was not suitable for RFP expression, possibly due to its adverse impact on the growth and physiology of the cells. YFP expression exhibited a middle trend where pQE30-based plasmid showed highest expression and other two plasmids showed only 2–5-fold lower expression. These expression patterns of the transcript was also found to be reflected at the protein level when the samples from induced culture were separated on SDS-PAGE and detected on Western-blot (Supplementary Figure S1).

We also assessed the florescence emission ability of each transformants under confocal scanning microscope. The number of cells fluorescing in the GFP transformants as well as intensity of fluorescence of these cells followed pQE30 > pAS01 > pAS02 pattern, as expected from their respective origin of replication and from the previous expression data (Fig. 4A). The RFP showed poor fluorescence from pQE30-based plasmid, while the medium and low copy vector exhibited relatively high fluorescence (Fig. 4B). This kind of unusual pattern was also obvious from the plasmid copy number and gene expression data. YFP on the other hand showed highest fluorescence for the pQE30-based plasmid and then a drastic drop in fluorescence for pAS01-based plasmid and a negligible fluorescence for pAS02-based plasmid (Fig. 4C). A general observation for GFP and YFP had been that their intensity of fluorescence reduced with the lower plasmid copy number, while the number of fluorescing cells remained more or less similar. On the other hand, in case of RFP, the number of fluorescing cells is much less for pQE30 while the intensity remained similar. This phenomenon indicates the issue of plasmid stability for pQE30-based construct bearing RFP gene (Fig. 4).

3.5. Multiple gene co-expression at three different expression levels

The three fluorescent protein encoding genes GFPuv, TurboRFP and EYFP, each cloned in three different plasmids having compatible origin of replications, were co-expressed at different levels depending on plasmid expression system generating six strains, i.e., GRY, Gyr, RGY, RYG, YGR and YRG, as mentioned in Materials and Methods Section 2.2 and Table 1. Here, the first, second and third position in the strain nomenclature denotes high (pQE30), medium (pAS01) and low (pAS02) copy plasmid and letter ‘G’, ‘R’ and ‘Y’ denotes GFPuv, TurboRFP and EYFP gene, respectively. Gene expression from the generated constructs was first analyzed using real-time qPCR.

All the three genes were found to be expressed in all the six strains by real-time qPCR (Fig. 5). The overall expression level of all the three genes across all the six strains seem not to be statistically different at
0.05 level (except for YGR vs YRG) (Supplementary Table S1). In RGY, RYG, YGR, and YRG strains, the gene expression levels were consistent with their plasmid copy numbers (Fig. 5A). However in GRY strain, the highest level of gene expression was for TurboRFP present in medium copy plasmid rather than for GFPuv that was present in high copy plasmid. Also in GYR strain, the EYFP gene expression was lowest even when it was present in the medium copy plasmid, while TurboRFP present in the lowest copy plasmid showed moderate expression. These results suggest that while in majority of the conditions, the co-expression of multiple genes follows the expected expression pattern, there are few occasions where the expected norms are not followed.

To understand better about the occasions where the norms were not followed, we tried to understand the impact of co-expression of proteins on each other. When the expression of each gene was analyzed across all the six strains, it was observed that the genes expressed via high copy plasmid led to highest transcript level, irrespective of whether the second gene is cloned in the medium copy or the low copy plasmid (Fig. 5B). With respect to expression pattern of genes present in medium copy plasmid, we found that both GFP and YFP showed higher expression when RFP was present in high copy as compared to any of the other two. For example, GFP showed 1.16-fold expression in the RGY strain where GFP was in the medium copy and RFP was in the high copy plasmid. On the contrary, GFP showed only 0.28-fold expression in the YGR strain where YFP was in high copy. Similarly, YFP expressed better when RFP was either in the medium or higher copy plasmid. None of the other two proteins had significant impact on RFP expression. Pearson correlations between these pairs of proteins performed based on qPCR data also suggested correlation in the expression. A highly significant positive correlation was found between YFP and RFP at the 0.01 level, while significant positive correlations were found between GFP and each RFP and YFP at the 0.05 level (Supplementary Table S2).

3.6. Quantification of fluorescence co-expression via cytometry

For accurate quantification and qualification of different fluorescent proteins in all strains, FACS was used to detect different fluorophores in each strain. The cells transformed with the pQE30, pASA01, and pASA02 backbone plasmids were used as negative controls. Each strain had its positive controls corresponding to its constructs. All the three fluorescence proteins, i.e., GFP, RFP and YFP, were detected in all the six strains via fluorescence microscopy (Fig. 6), with their intensity and numbers varied between the strains as expected.

As mentioned before, the high level expression of EYFP overshadowed GFPuv expression level due to some overlap in their emission spectra. This was obvious with 97.6% cells expressing GFPuv in GRY strain, while only 37.5% cells expressed GFPuv in GYR strain. Moreover, the number of cells producing GFPuv were found to be negligible in the cases where GFPuv was expressed in the low and medium copy plasmid and YFP was co-expressed in high copy, e.g., GYR and YRG (Fig. 7, Supplementary Figure S3). Most of the cells showed RFP expression in the high (>97% cells) and medium (77–99% cells) copy plasmid range, while ~30% of the cells showed RFP expression in the low copy plasmid transformants. In case of YFP, high number of cells (94–99% cells) showed expression in all combinations, except for GRY strain where ~40% of cells showed YFP expression (Fig. 7). In terms of intensity of these fluorescence proteins, the highest florescence were detected where these proteins were expressed via high copy plasmid (Supplementary Figure S4). However, importantly, a significant difference in intensity exist between the two strains carrying high copy plasmid for each protein (eg. GRY vs GYR for GFPuv; RGY vs RYG for TurboRFP and YGR vs YRG for EYFP) (Supplementary Figure S4). The highest intensity was registered by YFP in YRG strain, followed by RFP in RYG strain. As expected, Pearson correlations between all pairs of proteins performed based on FACS data indicated a highly significant positive correlation between all proteins (Supplementary Table S3).

Fig. 4. Analysis of expression for single plasmid transformants expressing each of the three traffic signal reporter proteins GFPuv (A), TurboRFP (B) and EYFP (C) in high copy pQE30, medium copy pASA01 and low copy pASA02 plasmid using confocal microscopy. The first column is merged picture of phase-contrast with fluorescent image, second column is fluorescence image and last column is phase contrast image only.
3.7. Plasmid heterogeneity and stability at the cellular level

Plasmid loss is the principle factor of decreased recombinant protein productivity in plasmid-based systems, thus plasmid stability is necessary for stable and precise production of recombinant proteins. Although, naturally found E. coli plasmids are extremely stable, engineered expression plasmids are sometime lost upon co-expressed with other vectors, causing significant reductions of protein yields [20]. The six bacterial strains (GRY, GYR, RGY, RYG, YGR, and YRG) were grown, induced, appropriate dilutions were made in LB media and equal volumes were plated on 4 different LB agar plates, one was plain LB-agar plate, while three were having three different antibiotics according to the antibiotic markers present in the three plasmid (Supplementary Figure S5). Standard plate count method was used to obtain colony-forming units (CFU) from each plate for all strains. The data showed that the highest number of colonies on the non-selective plate was in GYR combination with about 100 million cells per ml of the liquid culture, while the lowest was in RGY combination with about 2 million cells (Fig. 8A). This suggest that while GFP is very well tolerated by the cells in the high copy plasmid, RFP is deleterious to the cells in the high copy. Also, the cell populations declined to less than half when GFP in high copy was associated with RFP in medium copy (in GRY) as compared to the strain GYR where YFP was in the medium copy. Similarly, the cell populations declined to less than half when YFP in high copy was associated with RFP in medium copy (in case of YRG) as compared to the strain YGR where GFP was in the medium copy. Cells with RFP in high copy preferred YFP in medium copy rather than GFP in medium copy. We further looked at whether all the three plasmids were present in the 6-co-transformed strains by plating the cells on different antibiotic plates. It was found that the GFP containing plasmids were the most stable-ones (87 – 97% positive population) irrespective of their origin of replications (Fig. 8B). The RFP containing plasmids were least...
stable in high copy plasmid (44 to 60% positive population) and most stable in low copy plasmid (68–96% positive population). YFP exhibited 75% and 92% positive population in high copy and low copy plasmid, respectively. YFP in the medium copy exhibited diverse profile with 93% positive population in strain GYR having GFP in high copy and only 29% positive population in strain RYG when RFP was in high copy.

The overall observation was that the largest number of populations in all the six co-transformants were bearing GFP containing plasmid and thus was the most favorable protein for the *E. coli*. On the other hand, RFP containing plasmids were the least suitable for the cells, indicating its possible toxicity and growth-inhibitory effect to the cells.

Fig. 6. Fluorescent micrographs showing different fluorescent proteins with different expression levels in *E. coli*. Individual cells with green, red, and yellow fluorescent proteins are shown in their colors along with merged photos and negative control.
4. Conclusions

In this study, we designed a 3-plasmid system having compatible origin of replication leading to high, medium and low copy number plasmid. All three plasmids had same promoter, RBS, and terminator for uniformity in expression in terms of inducer used. We cloned three commonly used reporter genes in the lab, i.e., GFPuv, EYFP, and TurboRFP, in all three plasmids to generate nine constructs and evaluated...
their expression individually and in combination. The real time PCR and emission of fluorescence of the single plasmid transformed cells indicated that GFP and YFP were expressed best in the high copy plasmid, while RFP expression declined in the high copy plasmid. When all the three plasmids were transformed in six different combination in the E. coli host, it was found in many of them that the co-expression of proteins led to interference in expression on each other. Importantly, a significant loss of viability was observed for the strain RGY, suggesting TurboRFP may be imparting toxic effect to the cells when expressed in high copy. Overall, our toolkit allows the expression of multiple gene/operons in different proportion. Further, our study provides new insights on the co-expression of common reporter proteins in the laboratory.

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6. Author contributions

ASA and SSY designed the study, ASA performed experimental work, ASA and SSY analyzed the data and wrote the manuscript, SSY generated resources for the study.

Declaration of Competing Interest

Authors have no competing interests to declare.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2021.e00692.
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