

# Plasmids for independently tunable, low-noise gene expression

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# Abstract

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Some microbiology experiments and biotechnology applications can be improved if it is possible to tune the expression of two different genes at the same time with cell-to-cell variation at or below the level of genes constitutively expressed from the chromosome (the “extrinsic noise limit”). This was recently achieved for a single gene by exploiting negative autoregulation by the tetracycline repressor (TetR), and bicistronic gene expression to reduce gene expression noise. We report new plasmids that use the same principles to achieve simultaneous, low-noise expression for two genes. The TetR system was moved to a compatible plasmid backbone, and a system based on the lactose repressor (LacI) was found to also exhibit gene expression noise below the extrinsic noise limit. We characterized gene expression mean and noise across the range of induction levels for these strains, applied the LacI system to tune expression for single-molecule mRNA detection in two different growth conditions, and showed that two plasmids can be co-transformed to independently tune expression of two different genes.

# Introduction

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We recently reported the development of a plasmid-based gene-expression system in which a gene of interest is expressed bicistronically with the tetracycline repressor (TetR) [1]. Using this gene expression system, cell-to-cell is below the “extrinsic noise limit” observed for genes expressed from the chromosome [2]. When TetR and GFP are expressed bicistronically, GFP induction and gene expression noise is similar to that observed for a TetR-GFP fusion protein with autoregulation [3]. Compared to induction of gene expression under the control of a constitutively expressed transcriptional repressor, the inducer dose-response is relatively linearized, and gene expression noise is much lower at intermediate induction levels.

Why this would it be useful to do this for two different genes at the same time? Example for stoichiometric production of a protein complex Expressing genes at their native levels ... e.g. for generating chromosome deletions

Figure 1: Regulatory constructs used in this study. (a) pZH509, (b) pJS102, (c) pJS103, (d) pJS23103

## Methods

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### Strain construction

All plasmids were constructed using isothermal assembly [4] of fragments generated by PCR or double stranded DNA synthesis (IDT) and transformed Top10 E. coli cells (IBA #5-1600-020). Transformants were screened by colony PCR and verified by sequencing. Sequence maps are included as supplementary files and plasmids will be made available at AddGene after deposit and screening [5]. Purified plasmids were transformed into E. coli strain MG1655 by growing 2 mL of culture in SOB media at 37 C to OD600=0.4, washing twice with 1 mL ice-cold water, resuspending in 40  $\mu$ L water, electroporation of 1–10 ng plasmid with the EC1 setting of a Micropulser (BioRad), and recovering for 1 hour at 37 C in SOC media.

To generate pJS101 with a compatible backbone, plasmid pZH509 [1] was used as a template to amplify the bicistronic regulatory construct including the  $P_{\text{TetO-1}}$  promoter [6], GFPmut2 [7], tn10 TetR [8] and rrnB T1 transcription terminator [9]. This was assembled by isothermal assembly with the backbone from pGB2 [10] with the pSC101 origin of replication and spectinomycin resistance to generate plasmid pJS101. Plasmids with the pSC101 and p15a origins of replication have been co-transformed in previous synthetic biology experiments [11].

Plasmids pJS102 and pJS103 were generated by 3-fragment isothermal assembly. Plasmid pZH509 was used as a template both for the vector backbone and for GFPmut2, with non-homologous extensions added to PCR primers to generate the  $P_{\text{LlacO}}$  and  $P_{\text{LlacOsym}}$  promoters.

Promoter  $P_{LlacOsym}$  lacks the central G–C base pair in  $P_{LlacO}$  LacI binding sites, which are symmetrical [12] except that the second *lacOsym* sequence has one base changed to maintain the  $P_{LlacO}$  promoter –10 sequence. LacI was amplified from *E. coli* MG1655 by colony PCR. Plasmid pJS23103 was made by isothermal assembly of an inverse PCR product of pZH509, with non-homologous primer extensions adding the weak, constitutive BBa\_J23103 promoter between GFPmut2 and TetR [13].

Plasmids were co-transformed into MG1655 by electroporation following the above protocol, except with 1  $\mu$ L each undiluted plasmid (~20–40 ng) and selecting on plates with both spectinomycin and carbenicillin.

Table 1: Plasmids used in this study.

Plasmid	Ori	Promoter	GOI	Repressor Expression	Reference
pZH501	p15a	CI-SNAP-tag	$P_{LtetO-1}$	bicistronic	[1]
pZH509	p15a	GFPmut2	$P_{LtetO-1}$	bicistronic	[1]
pZH520	p15a	GFPmut2	$P_{LtetO-1}$	constitutive	[1]
pJS101	pSC101	GFPmut2	$P_{LtetO-1}$	bicistronic	This work
pJS102	p15a	GFPmut2	$P_{LlacO}$	bicistronic	This work
pJS103	p15a	GFPmut2	$P_{LlacOsym}$	bicistronic	This work
pJS23103	p15a	GFPmut2	$P_{LtetO-1}$	bicistronic/ constitutive	This work
pZH742	pSC101	PP7cp- mNeonGreen	$P_{LlacO}$	bicistronic	This work
pZH740	p15a	mScarlet- I-24xPP7sl	$P_{LtetO-1}$	constitutive	This work

## Cell Growth

Cells were grown in overnight cultures at 32 C in M9 minimal media supplemented with 1% SOB media, 50  $\mu$ g/mL carbenicillin or 50  $\mu$ g/mL spectinomycin. The next day, cells were diluted 1:100 or more (if multiple experiments were to be done sequentially over several hours) in the same media supplemented with anhydrotetracycline. In this growth condition, doubling time was approximately 30 minutes for MG1655 strains harboring these plasmids.

## Microscopy

Microscopy methods and microscopy data analysis methods

## Flow cytometry

Flow cytometry methods and data analysis methods

# Results

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## Moving bicistronic autoregulatory construct to a compatible plasmid backbone

Info and testing for pJS101

Info for pJS102 / pJS103

Figure 2: pZH509 vs pJS101 (a) mean(aTc), (b) noise(mean) flow cytometry

## Alternative regulatory constructs with LacI replacing TetR

We hypothesized that replacing  $P_{LtetO-1}$  with a promoter with similar characteristics and replacing TetR with a ...

Figure 3: pZH509 vs pJS102 vs pJS103 (a) mean(aTc), (b) noise(mean), (c) Microscope images at mid induction, (d) noise(mean) from microscope images

## Increasing dynamic range with the addition of a weak constitutive promoter

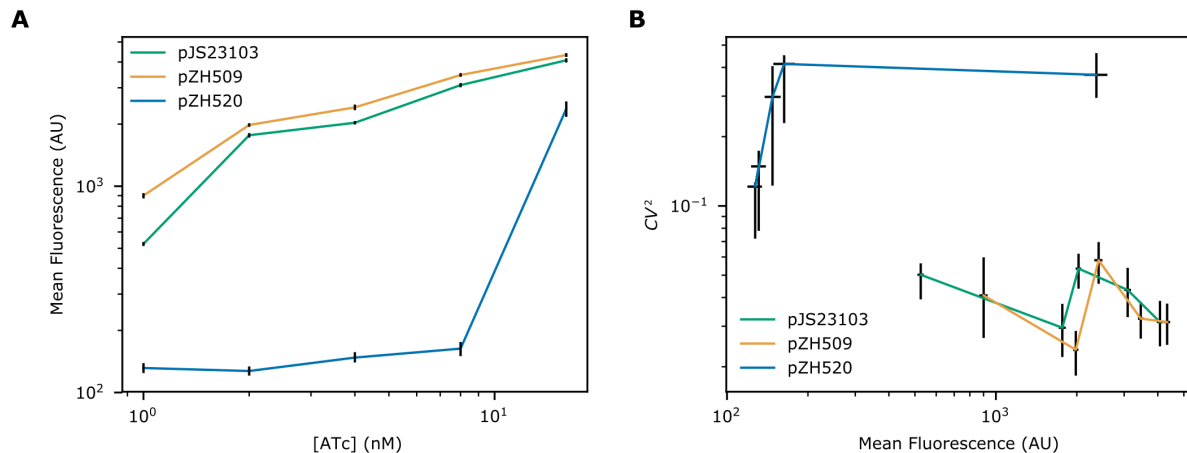


Figure 1: **(A)** Mean GFP fluorescence for pJS23103, pZH509 and pZH520 as a function of ATc concentration. The additional weak, constitutive promoter for TetR in pJS23103 results in lower GFP expression at low ATc concentrations. **(B)** Noise ( $CV^2$ ) as a function of mean GFP expression for the same strains. pJS23103 retains the low gene expression noise characteristic of pZH509. Error bars in both plots are bootstrapped 95% confidence intervals from a single experiment. The mean and variance of the pZH501 strain which does not express GFP was subtracted from each sample.

Figure 4: ZH509 vs pZH520 vs pJS23103 (a) mean(aTc), (b) noise(mean), (c) Microscope images at mid induction, (d) noise(mean) from microscope images

## Expression of two genes by remixing these components

Aggregation of mRNA-binding proteins from RNA bacteriophages makes it difficult to use fluorescent fusion proteins to detect single mRNAs [14]. We hypothesized that ...

[15] mNeonGreen [16] mScarlet-I [17] The Pf3 coat protein is a small membrane-spanning protein with a cytoplasm-facing C terminus.

## Discussion

Possible to improve pJS23103 with somewhat stronger promoter?

Possible to extend to 3 genes? Possible using additional repressor, pMB1 origin of replication, and different antibiotic selection.

## Author contributions

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JS and ZH designed experiments, performed experiments, analyzed data, and wrote the paper. ZH supervised the project.

## Acknowledgments

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