SUPPLEMENTAL INFORMATION

Expression optimization and synthetic gene networks in cell-free systems

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S1. Plasmid Descriptions

Tables S1-S6 below describe all plasmids used in this study, and construction methods are provided below each table. All plasmids in the tables were newly constructed for this study, with the exception of pDEST17-EGFP (50). Oligos were ordered from IDT DNA (Coralville, Iowa). All PCR products were run on agarose gels and purified with the QIAquick Gel Extraction Kit from Qiagen (Valencia, CA). Restriction enzymes were purchased from NEB (Ipswich, MA) and Fermentas (Glen Burnie, Maryland). Digestion products were purified with the QIAquick PCR Purification Kit from Qiagen. For ligations, T4 DNA ligase from NEB or Promega (Madison, WI) was used. Final constructs were sequenced for verification by Laragen, Inc (Culver city, CA).

Table S1: Constitutive test constructs

Plasmid	Promoter	RBS	Reporter	Backbone	Terminator
pT7	T7	ZE21	GFPmut3.1	pZE21-MSC-2	T1
pKSGFP	T7	g10	EGFP	pBluescript II KS (+)	T7
pDEST17-EGFP	T7	g10	EGFP	pDEST-17	T7

The plasmid pT7 was constructed by amplifying pT7tet by PCR with the following oligos:
5' AAGTGTCACCTGCATATACGGTTTCCCAGTACAGGACGCACTGACCGAATTC
5' AAGTGTCACCTGCATATCCGTTGTGGTCTCCCTATAGTGAGTCGTATTAGCGAAG
The PCR product was digested with AarI, and the purified digestion product was circularized by ligation.

To build pKSGFP, the *EGFP* coding sequence was amplified from pDEST17-EGFP using T7 promoter and T7 terminator primers. The PCR product and pBluescript II KS (+) were each digested with XbaI and EcoRV and ligated to form pKSGFP.

Table S2: TetR repressible constructs

Plasmid	Promoter	RBS	Reporter	Backbone	Terminator
pT7tet	T7tet13	ZE21	GFPmut3.1(ASV)	pZE21-MSC-2	T1
pT7tet2	T7tet19	ZE21	GFPmut3.1(ASV)	pZE21-MSC-2	T1
pT7tet-RBSII	T7tet13	RBSII	GFPmut3.1(ASV)	pZE21-MSC-2	T1
pT7tet-RBSg10	T7tet13	g10	GFPmut3.1(ASV)	pZE21-MSC-2	T1
pT7tet-RBS35	T7tet13	BBa_B0035	GFPmut3.1(ASV)	pZE21-MSC-2	T1
pT7tet-RBSA	T7tet13	A	GFPmut3.1(ASV)	pZE21-MSC-2	T1
pUC-T7tet	T7tet13	g10	EGFP	pUC-19	vsv
pUC-T7tet-T7term	T7tet13	g10	EGFP	pUC-19	T7
pT7tetKS	T7tet13	g10	EGFP	pBluescript II KS (+)	T7
pT7tetKS-ASV	T7tet13	g10	GFPmut3.1(ASV)	pBluescript II KS (+)	T7
pT7tetKS-SF	T7tet13	g10	Superfolder GFP	pBluescript II KS (+)	T7

To construct pT7tet, pGFP-ASV (49) was amplified by PCR using the following primers:
5' AAGTGTCACCTGCTGCAGTACAGGACGCACTGACCGAATTC
5' AAGTGTCACCTGCATCTGCGAAGGTGAAGACGAAAGGGCC
The PCR product was digested with AarI and was ligated to the following annealed oligos coding for the T7tet13 promoter:
5' TCGCTAATACGACTCACTATAGGGTCCCTATCAGTGATAGAGA
5' GTACTCTCTATCACTGATAGGGACCCTATAGTGAGTCGTATTA

To construct pT7tet2, pGFP-ASV was amplified by PCR using the same primers as for making pT7tet. The PCR product was digested with AarI and was ligated to the following annealed oligos coding for the T7tet19 promoter:
5' TCGCTAATACGACTCACTATAGGGAGATCGTCCCTATCAGTGATAGAGATCT
5' GTACAGATCTCTATCACTGATAGGGACGATCTCCCTATAGTGAGTCGTATTA

pT7tet-RBSII was constructed by amplifying pT7tet using the oligos below, digesting the PCR product with AarI, and circularizing the purified digestion product by ligation:
5' AACTGTCACCTGCATCTGGAGAAATTAAGCATGCGTAAAGGAGAAGAACTTTTC
5' AACTGTCACCTGCATCTCTCCTCTTTAATTCAATGAATTCGGTCAGTGCGTC

pT7tet-RBSg10 was constructed by amplifying pT7tet using the oligos below, digesting the PCR product with AarI, and circularizing the purified digestion product by ligation:
5' AACTGTCACCTGCATCTAGGAGATATACATATGCGTAAAGGAGAAGAACTTTTC
5' AACTGTCACCTGCATCTTCCTTCTTAAAGTTAATGAATTCGGTCAGTGCGTC

pT7tet-RBS35 was constructed by amplifying pT7tet using the oligos below, digesting the PCR product with AarI, and circularizing the purified digestion product by ligation:
5' AACTGTCACCTGCATCTAGGAGAATACTAGATGCGTAAAGGAGAAGAACTTTTC
5' AACTGTCACCTGCATCTTCCTCTTTAATCTCAATGAATTCGGTCAGTGCGTC

pT7tet-RBSA was constructed by amplifying pT7tet using the oligos below, digesting the PCR product with AarI, and circularizing the purified digestion product by ligation:
5' AACTGTCACCTGCATCTAAGGAGGAAAAAAATGCGTAAAGGAGAAGAACTTTTC
5' AACTGTCACCTGCATCTCCTTCTTTAATCTCAATGAATTCGGTCAGTGCGTC

To construct pUC-T7tet, pClpP was used as a parent plasmid. pClpP was constructed by first amplifying the pUC-19 vector using the following oligos:
5’ AAGTGTCACCTGCATATCTACTATCTGTTAGTTTTTTTCTTCCTCGCTCACTGACTCGC
5’ AAGTGTCACCTGCTTCTGCGACCCTATAGTGAGTCGTATTAACCGTCATCACCGAAACGC
To construct the insert for pClpP, a colony PCR of MG1655 *E. coli* was performed using the following oligos:
5’ AAGTGTCACCTGCATATTCGCTTAAAGAGGAGAAATTAAGCATGTCATACAGCGGCGAACGAGATA
5’ AAGTGTCACCTGCATATGTAGTTAGTGATGGTGGTGATGGTGATTACGATGGGTCAGAATCGAATCG
The two PCR products were digested with AarI, and the purified digestion products were ligated. Then, pClpP was amplified with the following oligos:
5’ AAGTGTCACCTGCATATGCGAACCACCATCACTAACTACTATCTGTTAG
5’ AAGTGTCACCTGCTTCTGTCTCTATCACTGATAGGGACCCTATAGTGAGTCGTATTAACC
To construct the insert for pUC-T7tet, pDEST17-EGFP was amplified with the follwing primers:
5’ AAGTGTCACCTGCATATAGACTCTAGAAATAATTTTGTTTAACTTTAAGAAG
5’ AAGTGTCACCTGCATATTCGCGTCTTATTTGTAGAGCTC
Finally, the pClpP and pDEST17-EGFP PCR products were digested with AarI, and the purified digestion products were ligated.

To construct pUC-T7tet-T7term, pUCT7tet was first amplified by PCR with the following oligos:
5’ AAGTGTCACCTGCATATTCGCTTCCTCGCTCACTGACTCGC
5’ AAGTGTCACCTGCATATGTACGTTAGTGATGGTGGTTCGCGT
The, to create the insert, pKSGFP was amplified by PCR using the following oligos:
5’ AAGTGTCACCTGCATATGTACCTGCTAACAAAGCCCGAAAGG
5’ AAGTGTCACCTGCTTCTGCGAAAGCTTGATACACCCGTCCTGT
The two PCR products were digested by AarI, purified, and ligated.

To construct pT7tetKS, pKSGFP was amplified using the following primers:
5’ AAGTGTCACCTGCATATCAGTGATAGAGACCGCTCTAGAAATAATTTTGTTTAAC
5’ AAGTGTCACCTGCATATACTGATAGGGACCCTATAGTGAGTCGTATTACGCG
The PCR product was digested with AarI, and the purified product was circularized by ligation.

To construct pT7tetKS-ASV, pT7tetKS was amplified using the following oligos:
5’ AAGTGTCACCTGCATATTCGCGGTTGATTCGAAGGCTGCTAAC
5’ AAGTGTCACCTGCACGACATATGTATATCTCCTTCTTAAAG
pGFP-ASV was amplified using the following oligos:
5’ AAGTGTCACCTGCATATTATGCGTAAAGGAGAAGAACTTTTCAC
5’ AAGTGTCACCTGCATATGCGAGGCTGCAGGAATTCGATATCAA
The resulting PCR products were digested by AarI, and the purified digestion products were ligated.

To construct pT7tetKS-SF, pT7tetKS was amplified using the following oligos:
5’ AAGTGTCACCTGCATATTCGCGGTTGATTCGAAGGCTGCTAAC
5’ AAGTGTCACCTGCATATAAAGTTAAACAAAATTATTTCTAGAGCGGT
pET-sfGFP (89)was amplified using the following oligos:
5’ AAGTGTCACCTGCATATCTTTAAGAAGGAGATATACATATGGGTCATCACCACCACCATC
5’ AAGTGTCACCTGCTTCTGCGAACCAGACTCGAGGGTACCTCAT
The resulting PCR products were digested by AarI, and the purified digestion products were ligated.

Table S3: LacI repressible constructs

Plasmid	Promoter	RBS	CDS	Backbone	Terminator
pLacOIDGFP	T7LacOID	g10	*EGFP*	pET3a	T7
pLacOGFP	T7LacO1	g10	*EGFP*	pET3a	T7

To build pLacOGFP, the *EGFP* coding sequence was amplified from pKSGFP and inserted into pET15b between NdeI and HindIII to form an intermediate construct pGFPpET15b. The pGFPpET15b plasmid was digested using BglII and HindIII, and the digestion product containing the T7 promoter-LacO-RBS-GFP-Terminator sequence was inserted into pET3a.

To construct pLacOIDGFP, the LacOID operator region was inserted into pLacOGFP using the following PCR primers:
Fwd OID primer: 5' AATTGTGAGCGCTCACAATTGAAATAATTTTGTTTAACTTT
revpET3a primer: 5' /Phos/CCCTATAGTGAGTCGTATTAATTTCGCGG

Table S4: Repressor expression constructs

Plasmid	Promoter	RBS	CDS	Backbone	Terminator
pT7LacI	T7	g10	Lac Repressor- Start codon changed from GTG to ATG	pET3a	T7
pET-TetRHis	T7	g10	*tetR*	pET-15b	T7

To construct pT7LacI, the coding sequence for lac repressor was amplified from pFNK-101 (90) and the start codon was mutated from GTG to ATG using the following PCR primers:
5' TGTATCTGATCATATGGTGAATGTGAAACCA
5' TCATCCTCACGGATCCGCAATTCCGACGTCATTGCG
Both the *lacI* PCR product and the pET3a vector were digested using NdeI and BamHI, and resulting products were ligated.

To construct pET-TetRHis, pET-sfGFP was amplified by PCR using the following primers:
5' AAGTGTCACCTGCATATCTACTAATGAGGTACCCTCGAGTCTG
5' AAGTGTCACCTGCTTCTACATGGTATATCTCCTTCTTAAAGTTAAAC
pTetRgfp was amplified using the following primers:
5' AAGTGTCACCTGCATATATGTCCAGATTAGATAAAAGTAAAGTG
5' AAGTGTCACCTGCATATGTAGTTAGTGATGGTGGTGATGGTGAGACCCACTTTCACATTTAAGTTG
The PCR products were digested with AarI, purified, and ligated.

Table S5: Bicistronic constructs

Feedback plasmid	promoter	Gene1 RBS	Gene1	Gene2 RBS	Gene2	Backbone	Terminator
pCtltetKS	T7	g10	*tetR*	g10	*EGFP*	pBluescript II KS (+)	T7
pLacI-GFP	T7	g10	*lacI*	g10	*EGFP*	pBluescript II KS (+)	T7
pGFP-LacI	T7	g10	*EGFP*	g10	*lacI*	pBluescript II KS (+)	T7

pCtltetKS was constructed by amplifying pKSGFP using the following primers:
5' AAGTGTCACCTGCATATCCGCTCTAGAAATAATTTTGTTTAAC
5' AAGTGTCACCTGCATATGAGCGGCCCTATAGTGAGTCGTATTACGCG
To create the insert, pTetRgfp was amplified by PCR with the following primers:
5' AAGTGTCACCTGCTAATGCTCTAGAAATAATTTTGTTTAACTTT
AAGAAGGAGATATACATATGTCCAGATTAGATAAAAGTAAAGTG
5' AAGTGTCACCTGCTTTAGCGGCTTAAGACCCACTTTCACATTTAAG
The resulting products were digested with AarI, purified, and ligated.

To construct pLacI-GFP, the *lacI* coding sequence and ribosome binding site were amplified from pT7LacI using the following primers:
XbaIbicisfwd: 5' CGGTTTCCCTCTAGAAATAATTTTGT
BamHIlacIrev: 5' TCATCCTCACGGATCCGCAATTCCGACGTCATTGCG
The *EGFP* coding sequence along with ribosome binding sites were amplified from pKSGFP using the following primers:
BamHIGFPRBSFWD: 5' TACGCAGTATGGATCCAGCTCCACCGCGGTGGCGGC
PBSKSHindiiibicisrev: 5' GGTCGACGGTATCGATAAGCTT
The PCR product containing the *lacI* coding sequence was digested with XbaI and BamHI, while the PCR product containing the coding sequence for *EGFP* was digested with BamHI and HindIII. pBluescript II KS (+) was digested with XbaI and HindIII and the products were then ligated and transformed.

To construct pGFP-LacI, the GFP coding sequence and ribosome binding site were amplified from pKSGFP using the following primers:
XbaIbicisfwd: 5' CGGTTTCCCTCTAGAAATAATTTTGT
SphIGFPbicisrev: 5' ATACTGCGTAGCATGCCAACCACTTTGTACA
The lacI coding sequence and ribosome binding site were amplified from pT7LacI using the following primers:
SphIlacIbicisfwd: 5' TACGCAGTATGCATGCAATAATTTTGTTTAAC
pET3aHindIIIBicisrev: 5' TCATCGATAAGCTTTAATGCGGTAGTT
The PCR product containing the *EGFP* coding sequence was digested with XbaI and SphI while the PCR product containing the coding sequence for *lacI* was digested with SphI and HindIII. pBluescript II KS (+) was digested with XbaI and HindIII and the products were then ligated and transformed.

Table S6: Negative feedback systems

Feedback plasmid	promoter	TetR RBS	GFP RBS	Reporter	Backbone	Terminator
pNFB-T7tet	T7tet13	ZE21	ZE21	GFPmut3.1(ASV)	pZE21-MSC-2	T1
pNFB-T7tetKS	T7tet13	g10	g10	EGFP	pBluescript II KS (+)	T7
placOLacIGFP	T7lacO1	g10	g10	EGFP	pET3a	T7

pNFB-T7tet was constructed by amplifying pTetRgfp using the following oligos:
5' AAGTGTCACCTGCTGCAGTACAGGACGCACTGACCGAATTC
5' AAGTGTCACCTGCATCTGCGAAGGTGAAGACGAAAGGGCC
The PCR product was digested with AarI, the purified digestion product was ligated to the annealed oligos below encoding the T7tet13 promoter:
5' TCGCTAATACGACTCACTATAGGGTCCCTATCAGTGATAGAGA
5' GTACTCTCTATCACTGATAGGGACCCTATAGTGAGTCGTATTA

pNFB-T7tetKS was constructed by amplifying pTetRgfp using the following oligos:
5' AAGTGTCACCTGCTAATGCTCTAGAAATAATTTTGTTTAACTTT
AAGAAGGAGATATACATATGTCCAGATTAGATAAAAGTAAAGTG
5' AAGTGTCACCTGCTTTAGCGGCTTAAGACCCACTTTCACATTTAAG
pKSGFP was amplified using the following oligos:
5' AAGTGTCACCTGCATATCCGCTCTAGAAATAATTTTGTTTAAC
5' AAGTGTCACCTGCATATGAGCGGTCTCTATCACTGATAGGGACCCTATAGTGAGTCGTATTACGCG
The PCR products were digested with AarI, and the purified digestion products were then ligated.

placOLacIGFP was constructed by amplifying the EGFP coding sequence from pKSGFP, amplifying the LacI coding sequence from pT7LacI, and inserting the EGFP and LacI products into pET15b between NdeI and HindIII sites to construct the intermediate plasmid placOLacIGFPpET15b. The placOLacIGFPpET15b plasmid was then digested using BglII and HindIII, and the digestion product containing the T7 promoter-LacO-RBS-LacI-RBS-GFP-Terminator sequence was inserted into pET3a.

S2. Temperature of Cell-Free Reactions

Although a cell-free reaction temperature of 37°C was recommended by the kit manufacturer for maximizing expression yield, we chose to perform reactions at 30°C. This was done to aid in proper protein folding. Figure S1 compares the expression dynamics of our benchmark construct at 30°C and 37°C. Fluorescence was significantly more intense at 30°C, most likely due to more efficient GFP folding (91,92).

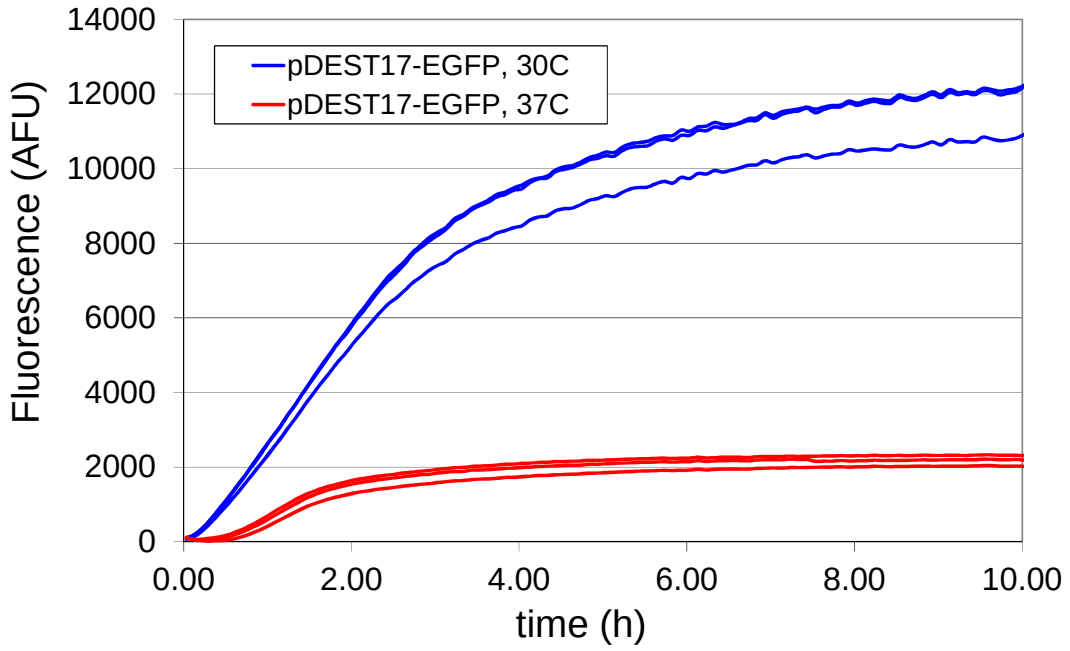

Figure S1: Dynamics of cell-free reaction using the Promega T7S30 High-Yield expression system and the benchmark plasmid pDEST17-EGFP. Triplicate results are shown for 30°C and 37°C.

S3. Reporter Experiments

We compared three different GFP variants: EGFP, GFPmut3.1(ASV), and superfolder GFP. The constructs used to compare these (pT7tetKS, pT7tetKS-ASV, and pT7tetKS-SF respectively) differed only in the reporter gene. EGFP gave the brightest fluorescence in our experiments.

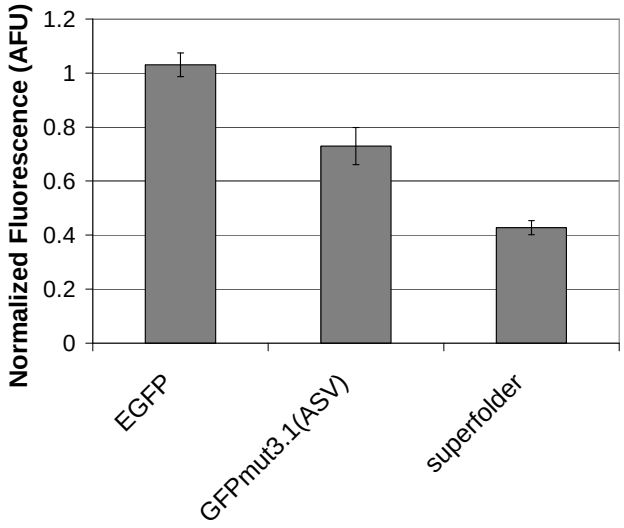

Figure S2: Comparison of EGFP, GFPmut3.1(ASV), and superfolder GFP using plasmids pT7tetKS, pT7tetKS-ASV, and pT7tetKS-SF respectively.

S4. DNA Concentration

For all cell-free expression experiments, we used the manufacturer recommended DNA concentration, 20 ng/μL. We tested the effect of DNA concentration on expression for several different constructs, and our results in Figure S3 support that this concentration is close to optimal for our constructs. As shown in this figure, additional amounts of DNA beyond 20 ng/μL do not result in increased fluorescence for our constitutive benchmark construct pDEST17-EGFP or for the TetR repressible constructs pT7tet-g10RBS and pUC-T7tet. For the negative feedback construct pNFB-T7tet, additional amounts of DNA beyond 20 ng/uL only slightly increased fluorescence under inducing conditions (1333 ng/mL aTc).

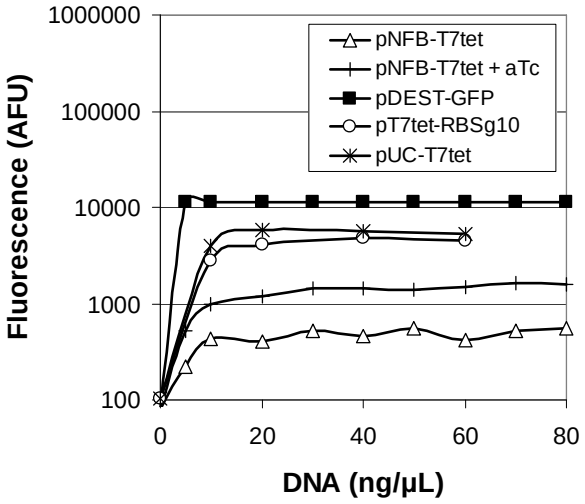

Figure S3: Effect of DNA concentration on expression efficiency for several constructs. Fluorescence was measured after 10 hours of expression at 30°C. To induce pNFB-T7tet, 1333 ng/μL aTc was used.

S5. Comparison of Multicistronic and Multi-Plasmid Approaches

To further compare the multicistronic and multi-plasmid approaches to system composition, we expressed the bicistronic constructs pLacI-GFP and pGFP-LacI in the concentrations shown in Figure S4. We also co-expressed pKSGFP and pLacI with each plasmid at the concentration shown in Figure S4. Thus, each location on the x-axis corresponds to an equal number of copies of the *EGFP* and *lacI* genes for pLacI-GFP, pGFP-lacI, and pKSGFP/pLacI. Significantly higher expression, as measured by fluorescence after 10 hours of expression, was realized with the bicistronic constructs.

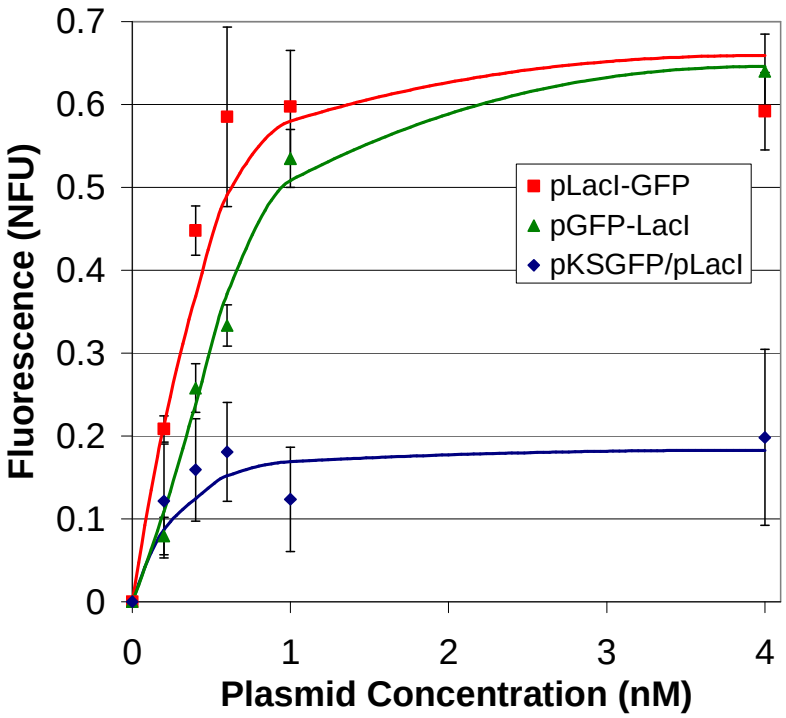

Figure S4: Comparison of bicistronic and two-plasmid systems. Normalized fluorescence after 10 hours of expression is shown for the bicistronic constructs pLacI-GFP and pGFP-lacI, along with the two-plasmid system pKSGFP/pLacI. Trendlines were generated in Matlab by smoothening the data using locally weighted scatterplot smoothing **(**LOWESS) with a span of 6, setting fluorescence to 0 at for the 0 nM plasmid concentration point, and then performing piecewise cubic Hermite interpolation.

S6. Decay Rates

EGFP is known to have a half-life of over 24 hours (Clontech), and its decay is insignificant over the duration of our experiments. On the other hand, mRNA typically has a faster decay time, and we sought to estimate the general decay rate of mRNA in the cell-free expression system. Although mRNA decay can depend on the transcript, many cell-free expression systems have been developed to maximize messenger lifetime.

To develop a general estimate of mRNA stability in the Promega T7S30 High-Yield system, GFPmut3.1(ASV) was expressed using the plasmid pFNK-503 (90) activated by the addition of 30 μM 3OC12HSL (11,88). Once fluorescence from this construct reached levels that were at least 10 times higher than background, 1 μg/mL rifampicin was added to inhibit further transcription. Fluorescence was measured every two minutes.

Assuming negligible EGFP decay and a stationary translation rate over the 1-2 hour duration of these experimental measurements, the dynamics of fluorescence following rifampicin addition at time t=0 should be described by Equations 1-2 below. Here, m is the mRNA concentration and p is GFP fluorescence, which we assume scales linearly with GFP protein concentration:

$$\frac{dm(t)}{dt} = -\gamma m(t) \qquad \text{Eq. 1}$$

$$\frac{dp(t)}{dt} = km(t) \qquad \text{Eq. 2}$$

Solving for $p(t)$ gives the following:

$$p(t) = p(0) + \frac{km(0)}{\gamma} - \frac{km(0)}{\gamma} e^{-\gamma t} \qquad \text{Eq. 3}$$

Equation 3 described the data well for all 10 experiments performed, as shown for one experiment in Figure S5. For each experiment, the initial fluorescence $p(0)$ was subtracted, and the data was fit to Equation 3 with p(0)=0. The mRNA half-life was then calculated as $\ln(2)/\gamma$, and the average from 10 experiments was 30min (std dev = 4min). This is an order of magnitude longer than typical mRNA degradation rates in live *E. coli* cells (93).

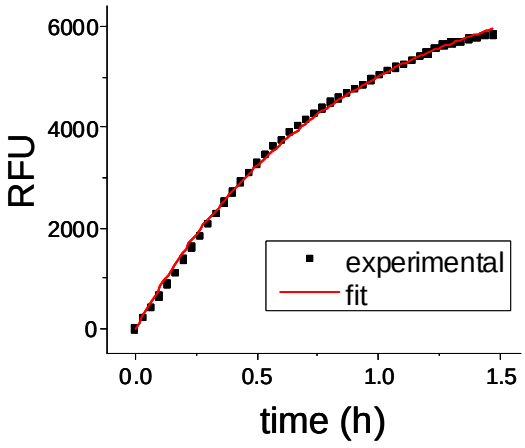

Figure S5: Experiments to determine mRNA degradation rate. Time 0 corresponds to the time at which rifampicin was added to inhibit further transcription. Fluorescence at time 0 was subtracted, and the data was fit to the equation $p(t) = (km(0)/\gamma)(1 - \exp(-\gamma t))$.

S7. Comparison of vsv and T7 terminators

To further compare the vsv and T7 terminators, we first examined transcripts produced from the pUCT7tet (vsv terminator) and pUCT7tet-T7term (T7 terminator) constructs using the Ambion MAXIscript T7 in vitro transcription kit. The upper main transcript band of the gel shown in Figure S6a corresponds to unterminated transcripts, while the lower main transcript band corresponds to properly terminated transcripts. No significant difference was revealed.

We then examined transcripts from these constructs using the Promega T7S30 High Yield expression system. As shown in Figure S6b, the only construct that yielded an observable band was circular pUCT7tet-T7term DNA (lane 4). The greater transcript yield and higher expression levels observed with the T7 terminator constructs can be partially explained by a lower mRNA degradation rate. To quantify the effect of the terminator sequence on mRNA stability, we used the approach presented in Section S6, except that 390 μM T7 lysozyme was used to inhibit transcription. Triplicate measurements revealed that the half-life of mRNA from the T7 terminator construct pUCT7tet-T7term was 28% longer than the half-life of mRNA from the vsv construct pUCT7tet, as shown in Figure S6c.

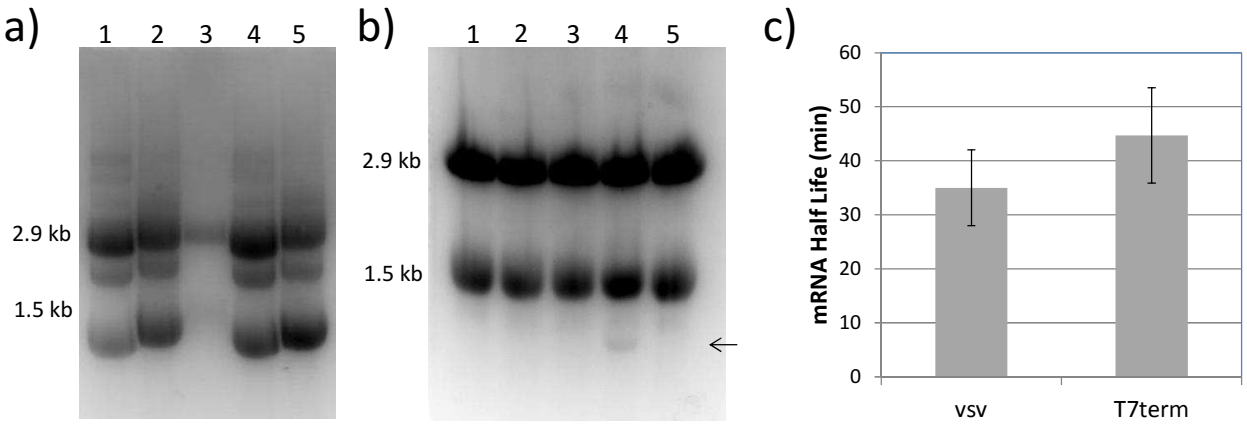

Figure S6: Comparison of vsv and T7 terminators. a) Transcripts from pUCT7tet (vsv) and pUCT7tet-T7term (T7) produced using the Ambion MAXIscript T7 kit. Lanes 1 and 4 are transcripts from pUCT7tet-T7term, lanes 2 and 5 are transcripts from pUCT7tet, and lane 3 is 23S and 16S rRNA. b) Transcripts produced using the Promega T7S30 High Yield kit. Lane 1 is a control consisting of cell extract with no DNA added. Lane 2 transcripts are from linearized pUCT7tet-T7term DNA. Lane 3 transcripts are from linearized pUCT7tet DNA. Lane 4 transcripts are from circular pUCT7tet-T7term DNA. Lane 5 transcripts are from circular pUCT7tet DNA. The dark upper band is 23S rRNA, and the dark lower band is 16S rRNA. c) Half-lives of vsv terminator and T7 terminator transcripts. Error bars represent standard deviation from three measurements.

S8. Negative Feedback with the Lac Repressor

We primarily focused on optimizing a TetR repressible T7 promoter and building feedback with this promoter, as Tet repressible T7 promoters have not been previously characterized. Also, the Promega T7S30 High Yield kit, which was used for all cell extract experiments, has IPTG pre-added. This precludes proper characterization of the Lac constructs. However, to demonstrate that feedback based on T7 transcription works with the Lac system, we constructed a negative feedback plasmid (placOLacIGFP described in Table S6) using the T7lacO1 promoter shown in Figure 2. Results in live BL21-AI cells (Invitrogen) are shown below. The control constructs pLacOGFP (Table S3) and pLacI-GFP (Table S5) exhibited no increase in fluorescence in response to IPTG, but the feedback construct placOLacIGFP showed five-fold induction with the addition of 30 µM IPTG. Further optimizations could be performed to improve the dynamic range of induction, such as the testing of different T7lac promoter variants and optimization of ribosome binding sites.

The plasmids pLacOGFP, pLacI-GFP, and placOLacIGFP were each transformed into chemically competent BL21-AI *E. coli*. Single colonies from each transformation plate were inoculated into 2 mL LB media with 100 µg/mL ampicillin and grown to log phase at 37ºC with shaking at 250 RPM. The cultures were then diluted 1:100 into 2 mL of M9 media (recipe described in Materials and Methods) with 100 µg/mL ampicillin and grown to log phase at 37°C with shaking. These cultures were diluted to an OD of ~0.01, and 1 mM L-arabinose was added to induce the expression of T7 polymerase. Aliquots (200 µL) were then dispensed into the wells of a 96-well plate (Corning 3370). The inducer IPTG was added as indicated in Figure S7. 50 µL of mineral oil was added to prevent evaporation. Plate reader measurements were taken as described in Materials and Methods, and the measurements in Figure S7 were taken after 2 hours of growth. Fluorescence values were corrected for background fluorescence, and absorbance readings at 600 nm were used to normalize for cell density. The resulting values were then divided by the fluorescence/absorbance measurement for the pLacOGFP sample with no IPTG.

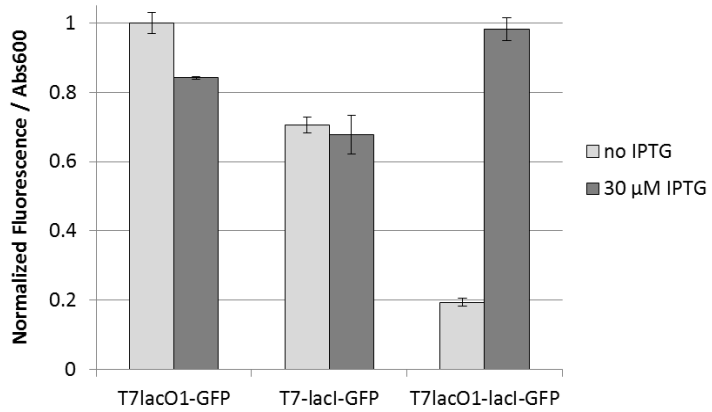

Figure S7: Control T7lacO1-GFP construct (pLacOGFP), control T7-lacI-GFP construct (pLacI-GFP), and T7lacO1-lacI-GFP negative feedback construct (placOLacIGFP) in live BL21-AI *E. coli* grown with 1 mM arabinose and IPTG as indicated. Error bars depict standard deviation of four measurements.

S9. Fluorescence of Inducer aTc

We verified that the fluorescence of the aTc inducer is insignificant compared to the intensity of GFP fluorescence produced from the feedback constructs. As shown in Figure S8, the normalized fluorescence value of the highest aTc concentration used in this study (3300 ng/mL) was 0.004. This is over an order of magnitude less than the fluorescence of the fully induced feedback constructs.

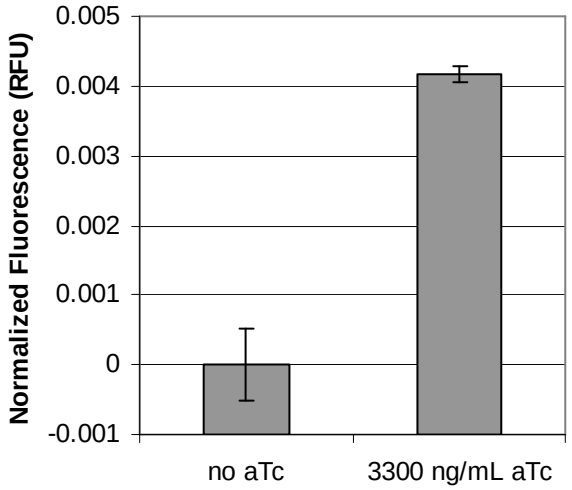

Figure S8: Normalized fluorescence of reaction mix with no DNA and reaction mix with no DNA and the highest aTc concentration used in this study, 3300 ng/mL. Readings were taken after 10 hours of incubation with shaking at 30°C.

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