Supporting Data:

Modular Control of Multiple Pathways using Engineered Orthogonal T7 Polymerases

I. Phage RNAP and Promoter Subfamilies

Bioinformatics was applied to guide our design of orthogonal RNAP:promoter interactions. Initially, we searched the NCBI database for homologs of the T7 RNAP via a BLAST search. This resulted in a set of 43 proteins with an E-value lower than 1^{-100}. This was narrowed to 28 by constraining the set to only include phage RNAPs with fully sequenced genomes. These were aligned using ClustalW to identify the specificity loop (T7 G732-P780) of each polymerase (Figure S1) (1). The 28 RNAPs were clustered into 13 subfamilies according to the ClustalW guide tree (family defined as < 0.1 distance between members).

Promoters in each phage genome were identified from annotations in the NCBI record. The list of identified promoters was supplemented by scanning each genome using the PHIRE (Phage *In silico* Regulatory Elements) software package (2). PHIRE performs algorithmic string-based search across a phage genome to identify regulatory elements based on conserved sequence similarity. WebLogo was used to generate sequence logos for each phage from the identified promoters (3), and the regions from -12bp to -7bp in each logo were aligned. Within the 13 identified phage subfamilies, the consensus promoter sequences for each phage are identical (Figure S1). A representative logo sequence is shown for each subfamily.

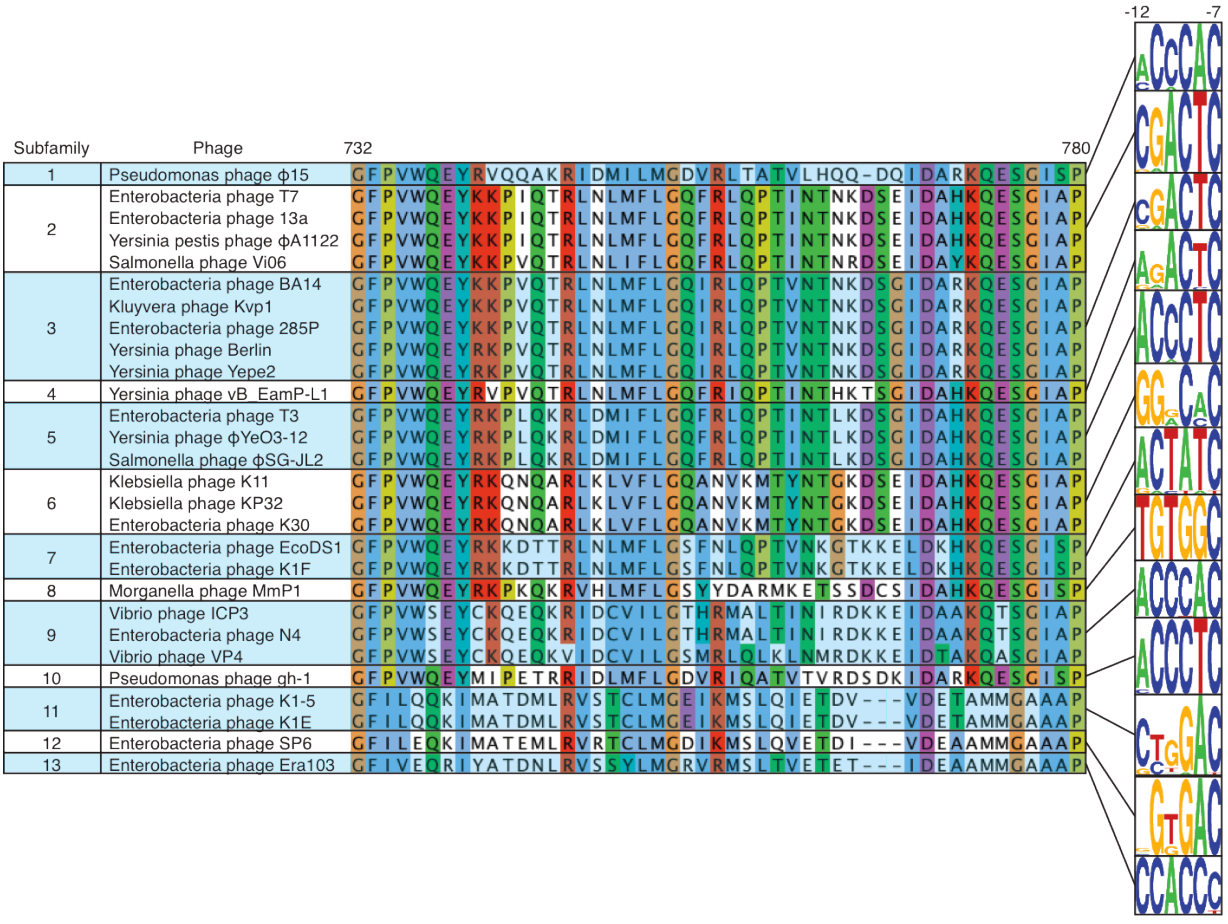

Figure S1: **RNAP subfamilies and associated promoter alignments.** T7-like phages are grouped into subfamilies based on sequence similarity of the RNAP specificity loop (G732-P780). Weblogo sequences were generated from identified promoters in each phage genome. Within each phage subfamily, consensus promoter sequences were consistent. A representative weblogo is shown for each subfamily.

II. Promoter Characterization

II.A. Relative Expression Units

In this manuscript, the output of promoters is reported as relative expression units (REU). This is simply a linear factor that is multiplied by the arbitrary units measured by the flow cytometer. The objective of normalizing to REU is to standardize measurements between labs and projects. The linear factor is 1.6×10^{-4} and the division by this number back converts to the raw arbitrary units. This number was calculated to be a proxy to the RPU (relative promoter units) reported by Kelly and co-workers (4). Our original standardized measurements involved a different reference promoter, fluorescent protein (mRFP), RBS, and plasmid backbone. Because of these differences, one cannot calculate RPU as defined by Kelly, et al. Instead, a series of plasmids was made (Figure S2) to estimate the relative expression of reporter protein from experimental constructs compared the standard construct in Kelly, et al. Conversion factors between constructs were measured and multiplied to obtain the linear factor above. We renamed the unit to REU (relative expression units) because it is intended to be a simple normalization of fluorescent units (akin to a fluorescent bead) and not a direct measurement of the activity of a promoter (*e.g.*, the polymerase flux). Portions of this methods section have been previously published and reproduced with permission (5).

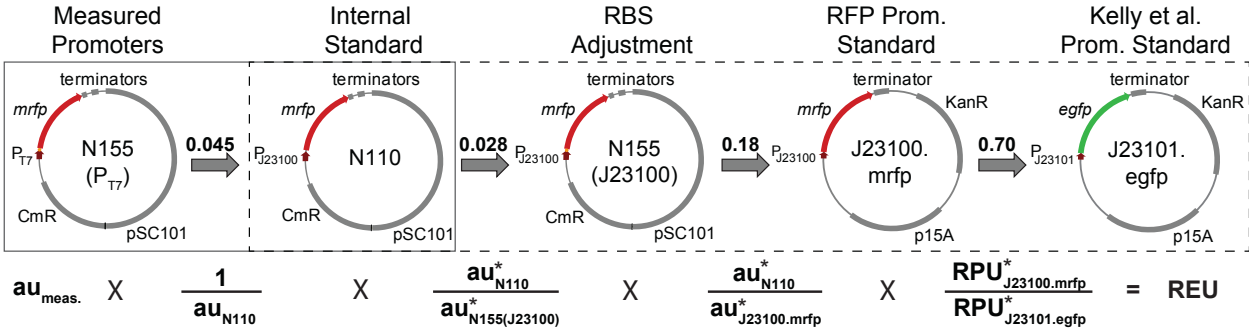

Figure S2. Promoter Characterization using Relative Expression Units. Conversion of arbitrary units into Relative Expression Units (REU). Promoters were characterized using mRFP1 fluorescent reporter protein in N155 (Measured Promoters). Data were first normalized by the fluorescence of N110 (Internal Standard) and then scaled by the fluorescence of N155(J23100) to account for RBS differences between N155 and N110 (RBS Adjustment). To directly compare our measurements to expression levels of the Kelly et al. standards, we further multiplied by the ratio of N110 fluorescence to the fluorescence of a Kelly standard plasmid expressing mRFP1 (RFP Promoter Standard). A final conversion factor is applied to compare all measurements to the Kelly et al. J23101-EGFP promoter standard based on a strong linear correlation of promoter strength (RPU) between constructs expressing mRFP and EGFP. Solid and dashed boxes were drawn to indicate which plasmids were measured at different facilities. Asterisked and non-asterisked units were measured in different facilities and correspond to the conversion factors directly above.

II.B. Inducible Promoter Characterization and T7 Promoter library

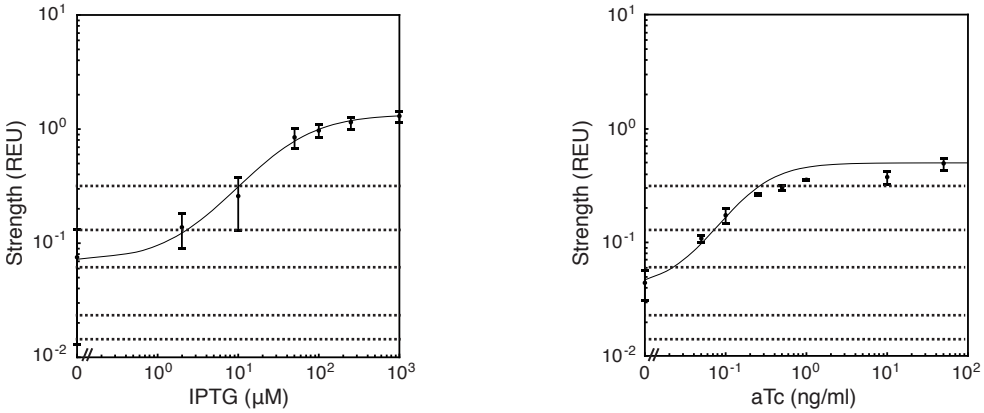

Figure S3. Promoter characterization for P_{tac} promoter (left) and P_{tet} promoter (right). The promoter strengths of P_{tac} promoter (plasmid N149) and P_{tet} promoter (plasmid N521) were measured under varied concentrations of inducers (IPTG or aTc). Each inducible promoter drives expression of mRFP in these constructs. The strengths of T7 promoters (T7 wild-type and mutants, Figure 3B) are shown as horizontal dotted lines. The strengths of T7 promoter mutants were characterized under 1mM IPTG induction from T7* RNAP (plasmid N249). The measured fluorescence (AU) for each mutant was converted to REU after subtracting the autofluorescence of cells not carrying any plasmid.

II.C. Detailed Promoter Orthogonality Measurements

Data is shown in Figure S4 corresponding to Figure 2B in the main text. This data provides the comparison of induced and uninduced fluorescence levels as well as error bars.

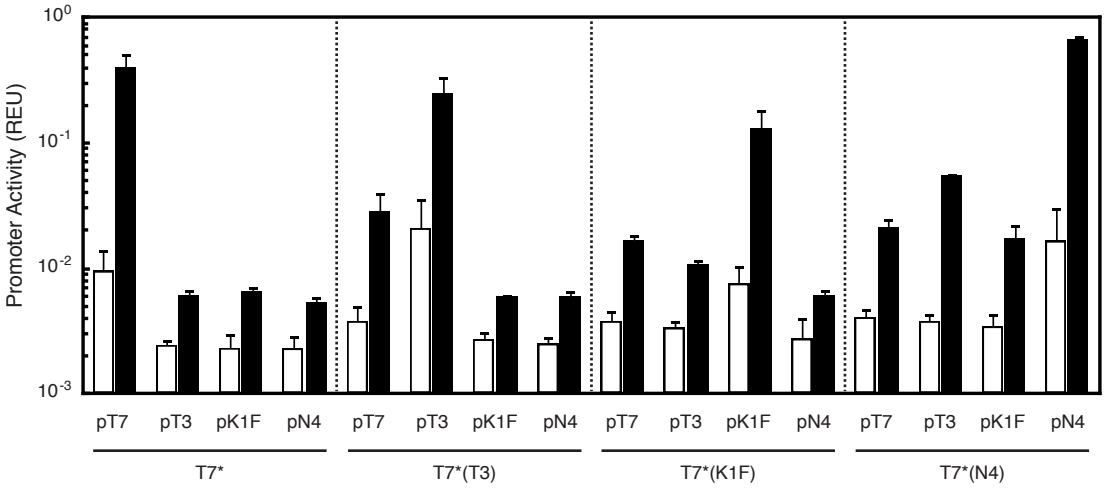

Figure S4: Orthogonality of all RNAP-promoter pairs. Orthogonal RNAPs were co-transformed with promoter mutants controlling mRFP in the base plasmid N155. The strength of expression under 1mM IPTG induction was measured by flow cytometry (geometric mean) and converted to relative expression units (REU) after subtraction of the autofluorescence of E coli harbouring no plasmid. Error bars represent the standard deviation of three experiments on different days.

II.D. Correlation of T7 and T3 Promoter Activity

Figure S5 shows the correlation between the T7 and T3 promoters that result from changing the strength-determining region. The strength of the T3 promoter is systematically higher, but the rank order is highly correlated.

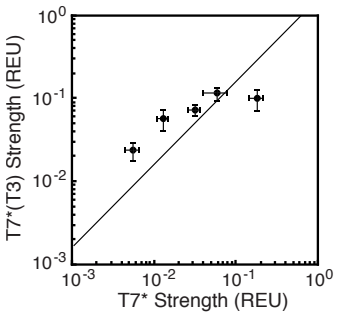

Figure S5: Modularity of strength region in the T7 promoter. The strengths of T7 promoters (Figure 3A, top left) are plotted against the strengths of the corresponding T3 promoters (Figure 3A, bottom right). Vertical error bars represent the error in measurement of T7 promoter strength taken from Figure 3A, and horizontal error bars reflect the T3 promoter strength error from Figure 3A. A line with slope of 1 is fit to the data.

II.E. Time Courses for of all the Orthogonal RNAPs

The timing of induction for each RNAP and its cognate promoter is shown in Figure S6. Briefly, cells were grown as described in the Fluorescence Characterization assay (Materials and Methods) with the following exceptions. Following the initial 14 hour incubation, cells were diluted into 5ml fresh LB (+ antibiotics, - inducers, final OD_{600} of 0.25, 15 ml Falcon tubes) and were incubated for 2.5 hours (37°C, 250 rpm). After this dilution step, cells were diluted into fresh LB a second time as described in the original assay. Timepoints are measured relative to the second dilution.

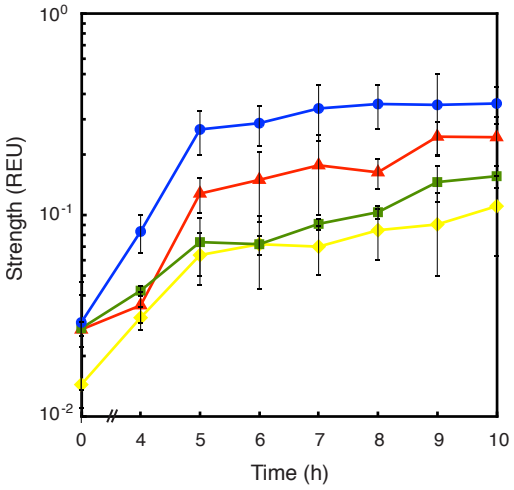

Figure S6: Temporal induction of RNAP expression. The expression of T7* RNAPs was measured over time. Orthogonal RNAPs were co-transformed with promoter mutants controlling mRFP in the base plasmid N155, and strength of expression under 1mM IPTG induction was measured by flow cytometry (geometric mean) and

converted to relative expression units (REU). Blue: T7*-P_{T7}, Green: T7*(T3)-P_{T3}, Yellow: T7*(K1F)-P_{K1F}, Red: T7*(N4)-P_{N4}. Error bars represent the standard deviation of three experiments on different days.

II.F. Representative Cytometry Distributions for the Orthogonal RNAPs

Representative population distributions from flow cytometry data for Figures 2, 3 and 4 are shown in Figure S7. Data were gated by forward and side scatter, and each dataset consists of at least 25,000 cells. Histograms correspond to cell counts binned by measured RFP fluorescence.

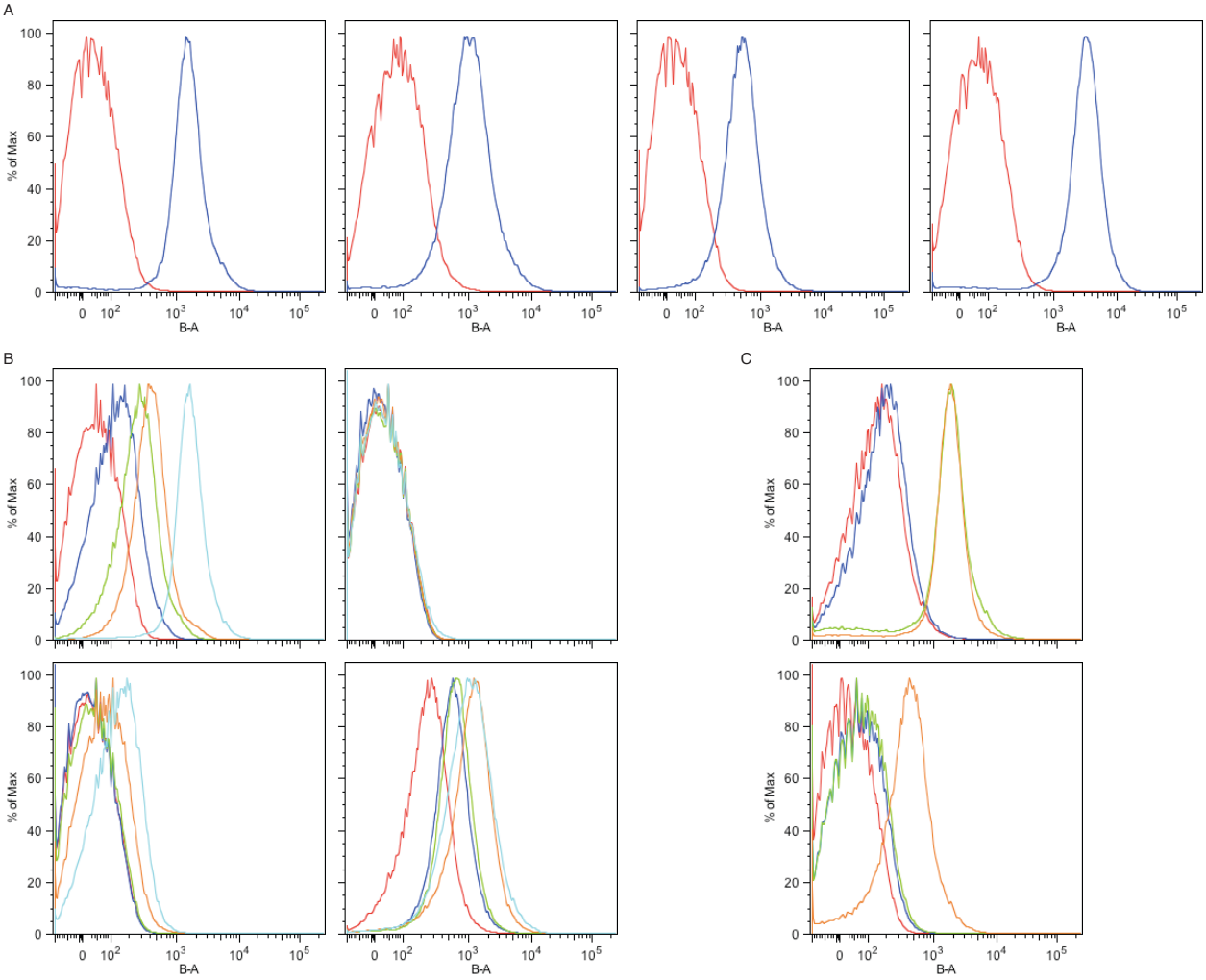

Figure S7: **Representative cytometry distributions. (A)** Cell distributions for uninduced (red) and induced (blue) populations carrying each RNAP and cognate promoter. Left to right: T7*-P_{T7}, T7*(T3)-P_{T3}, T7*(K1F)-P_{K1F}, T7*(N4)-P_{N4}. The distributions are representative samples from the data used to generate Figure 2C and Figure S4. **(B)** Cell distributions from the data used to generate Figure 3B. Colors corresponding to each promoter: 1, red; 2, blue; 3, green; 4, yellow; WT, cyan. **(C)** Cell distributions corresponding to Figure 4A (top) and Figure 4B (bottom). Colors correspond to inducing conditions as follows: none, red; aTc, blue; IPTG, green; both, yellow.

III. Terminator Characterization

Terminator strength is measured using the reporter plasmid shown in Figure S8. It contains GFP and mRFP, separated by the terminator to be measured. We eliminated the terminator to produce a control plasmid (N287) to serve as a reference for the calculation of strength. A T7 terminator library was cloned into the reporter plasmid and cotransformed with plasmid N249. Cells were grown under 1mM IPTG induction of T7* RNAP and screened for efficient termination by measuring reduction in mRFP expression via flow cytometry. GFP expression was also measured, and terminators were eliminated that exhibited more than 25% change in GFP expression from the control plasmid. In the screen, a putative strong terminator (T.8) was identified, but this was not included in the final set because there was no detectable hairpin structure. A library of 7 mutant terminators was characterized (Figure S9). From these measurements, normalized fluorescence values are calculated by dividing the mutant terminator fluorescent readings by the control plasmid fluorescent readings, *eg.* Normalized RFP = Terminator RFP / Control RFP. The strength of the terminator can be calculated as follows,

$$Termination\ Strength\ = {Normalized\ GFP\ (AU)}\Big/{Normalized\ RFP\ (AU)}$$

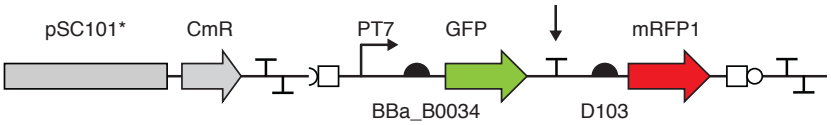

Figure S8: **Plasmid used to calculate terminator strength.** The fluorescent reporters, GFP and mRFP (6), are expressed under control of the T7 promoter and RBSs BBa_B0034 and D103. SBOL graphical notation is used to describe genetic parts: the BioBrick prefix and suffix are open squares, and terminators are in the shape of a *T.* The vertical arrow indicates location of the T7 terminator. Mutant terminators are cloned in place of the T7 terminator for characterization.

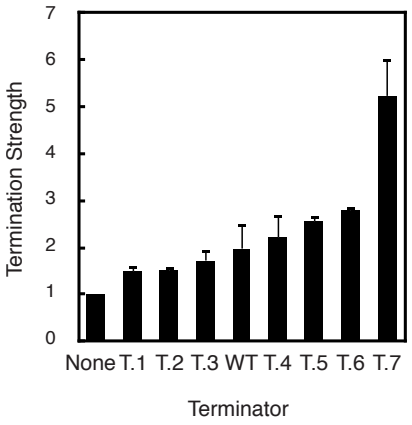

Figure S9. Terminator characterization. Terminator plasmids were co-transformed with plasmid N249 and characterized under 1mM IPTG induction of T7* RNAP. Data reported as fold knockdown and are the results of three experiments performed on multiple days.

IV. Plasmid Construction and Maps

Plasmids were designed *in silico* and constructed using standard DNA manipulation techniques. Assembly methods followed published protocols and included BioBrick (7), Megawhop (8), Phusion Site-Directed Mutagenesis or Gibson Assembly methods (9). Plasmids and parts located in the SynBERC Registry are referred to by their identification numbers, *e.g.* SBa_000XYZ.

Core Vectors
Plasmid pIncW (pSa, SpR) was generated from pEXT21 (pSa, SpR) by deletion of *osa*, *nuc1*, the Tn21 integrase gene, and ORF18 (10).

Plasmid pSB4C5 (pSC101, CmR) was obtained from the Registry of Standard Biological parts and serves as the base vector T7 promoter plasmids (7).

Plasmid pSB3K3 (p15a, KmR) was obtained from the Registry of Standard Biological parts and serves as the base vector for some pigment genes (7).

Empty Vectors for Toxicity Characterization
Plasmid N23 (pSC101, CmR) contains RBS BBa_B0034 and the *nifA* gene inserted between the BioBrick prefix and BioBrick suffix of pSB4C5.

Plasmid N84 (pIncW, SpR) contains a constitutive promoter (SBa_000565), a strong RBS (SBa_000475), and mRFP (SBa_000484) between the BioBrick prefix and BioBrick suffix of pIncW.

Standard Characterization Vectors
Plasmid N110 (SBa_000564) was constructed by inserting a constitutive promoter (SBa_000565), a strong RBS (SBa_000475), and mRFP (SBa_000484) between the BioBrick prefix and BioBrick suffix of pSB4C5.

Plasmid N292 (SBa_000566) was generated by inserting a terminator characterization cassette between the BioBrick prefix and BrioBrick suffix of pSB4C5. The cassette consists of the PT7 promoter, RBS (SBa_000498), GFP, the wild-type T7 terminator, RBS D103 (SBa_000563) from Salis *et. al.* (6), and mRFP (SBa_000484).

Plasmid N149 (SBa_000516) was constructed by inserting the P_{tac} promoter cassette (SBa_000563), RBS D103 (SBa_000563) from Salis *et. al.* (6), and mRFP (SBa_000484) between the BioBrick prefix and BioBrick suffix of pSB4C5.

Plasmid N521 (SBa_000567) was constructed by inserting a weak P_{tet} promoter cassette (SBa_000574), RBS D103 (SBa_000563), and mRFP (SBa_000484) between the BioBrick prefix and BioBrick suffix of pSB4C5.

Plasmid N155 (SBa_000568) was constructed by replacing the P_{tac} promoter cassette in N149 with the consensus T7 promoter sequence. The initially transcribed region from the P_{tac} promoter was retained (ggtaccaattgtgagcggataacaatt).

Plasmid N489 (SBa_000521) was constructed by deleting the initially transcribed region of the P_{tac} promoter from N155.

T7 RNAP Variants*
Plasmid N77 (SBa_000569) was constructed by inserting the P_{tac} promoter cassette (SBa_000563) and the wild-type T7 RNAP gene into pIncW.

Plasmid N115 (SBa_000570) was constructed by adding an N-terminal degradation tag (SBa_000509, TTGTTTATCAAGCCTGCGGATCTCCGCGAAATTGTGACTTTTCCGCTATTTAGCGATCTTGTTCAGTGTG GCTTTCCTTCACCGGCAGCAGATTACGTTGAACAGCGCATCGATCTGGGTGGC) to the T7 gene in N77.

Plasmid N219 (SBa_000571) was generated through single bp mutation (C1894A) in the T7 gene in N115.

Plasmid N249 (SBa_000520) was generated from N219 by placing the T7 gene under control of a weak RBS (SBa_000507, TATCCAAACCAGTAGCTCAATTGGAGTCGTCTAT), an insulator (SBa_000508), and changing the start codon from ATG to GTG.

Orthogonal RNAP Variants
Plasmid N616 (SBa_000572) was constructed by placing the K1F* RNAP under control of a weak P_{tet} promoter cassette (SBa_000574), in plasmid pIncW.

Plasmid N636 (SBa_000573) was generated by cloning the region between the BioBrick sites in plasmid N616 into the N249 BioBrick prefix. A DNA region containing bidirectional terminators was inserted between the N616 sourced DNA and the N249 DNA between the BioBrick sites.

Pigment Vectors
Plasmid N520 (SBa_000575) was derived from pAC-LYC by transferring the *crtEBI* operon to pSB3K3 (11).

Plasmid N537 (SBa_000576) was constructed through combinatorial assembly of 5 gene expression cassettes comprising the genes *dxs*, *idi*, *crtE*, *crtB* and *crtI* (12). Each gene expression cassette was created *in vitro* by joining an insulator sequence, a library of T7 promoters, a synthetic RBS, the gene of interest and a T7 terminator.

Plasmid N538 (SBa_000577) consists of the *vioABCDE* operon from pVIO4 under control of the wild-type T7 promoter in pSB4C5 (13).

Plasmid N554 (SBa_000578) was derived from N538 by replacing the T7 promoter with a K1F* promoter.

Plasmid N571 (SBa_000579) was derived from N554 by inserting a T7 terminator, insulator and K1F* promoter between *vioA* and *vioB*.

Plasmid N588 (SBa_000580) was derived from N571 by deleting *vioC* and *vioD*.

Plasmid N676 (SBa_000581) was derived by cloning the region between the BioBrick sites in N588 into pSB3K3.

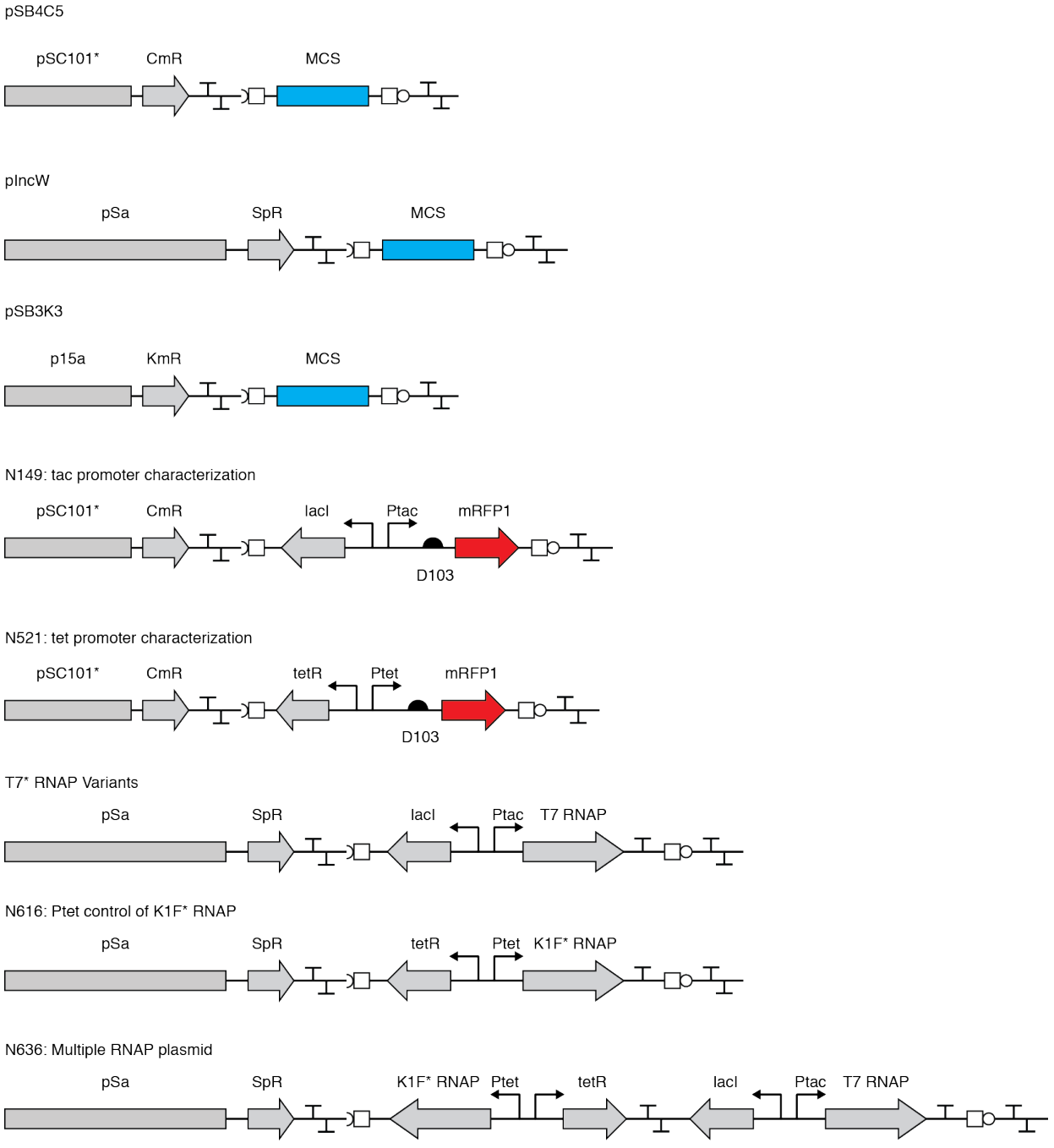

Figure S10: **Plasmid sequence maps.** Abbreviations: CmR, chloramphenicol resistance gene; SpR, spectinomycin resistance gene; KmR, kanamycin resistance gene; pSa, p15a, and pSC101* are origins. SBOL graphical notation is used to describe genetic parts: the BioBrick prefix and suffix are open squares, MCS refers to a generalized multi-cloning site surrounded by the BioBrick prefix and suffix, RBSs are solid half-circles, and terminators are in the shape of a *T*.

V. Part Sequences

Table S1. DNA sequences of synthetic parts

Part Number	Part Type	Sequence
SBa_000443	WT T7 Promoter	taatacgactcactatagggaga
SBa_000444	T7 Promoter 1	taatacgactcactacaggcaga
SBa_000445	T7 Promoter 2	taatacgactcactagagagaga
SBa_000446	T7 Promoter 3	taatacgactcactaatgggaga
SBa_000582	T7 Promoter 4	taatacgactcactataggtaga
SBa_000450	WT T7 Terminator	tagcataaccccttggggcctctaaacgggtcttgaggggttttttgt
SBa_000583	T7 Terminator 1	tacctcaaccccttccgccctcatatcgcggggcatgcggttttttgt
SBa_000584	T7 Terminator 2	taccctaaccccttccccggtcaatcggggcggatgggggttttttgt
SBa_000585	T7 Terminator 3	tagaccaaccccttgcggcctcaatcgggggggatgggggttttttgt
SBa_000452	T7 Terminator 4	tactctaaccccatcggccgtcttagggggttttttgt
SBa_000586	T7 Terminator 5	tagcagaaccgctaacgggggcgaagggggttttttgt
SBa_000451	T7 Terminator 6	tacatatcgggggggtaggggttttttgt
SBa_000587	T7 Terminator 7	tactcgaaccccctagcccgctcttatcgggcggctaggggttttttgt
SBa_000588	T7 Terminator 8	tataaaacgggggggctaggggttttttgt
SBa_000589	K1F rbs	aattgtgagcggataacaa
SBa_000592	dxs rbs	aagacgaaggaggccgcta
SBa_000594	idi rbs	ggttacaaggggaaaacga
SBa_000596	crtE rbs	gaataatccattaaaaggagggtataa
SBa_000598	crtB rbs	cacccgagtaaagagggagtaaactt
SBa_000600	crtI rbs	acccaatttattagaggagaaaactac
SBa_000606	vioA rbs	attaaagaggagaaattaagc
SBa_000593	idi insulator	gtctgtagcacgtgcatc
SBa_000595	crtE insulator	acacaatcttcctgctca
SBa_000597	crtB insulator	tccacgaacacagggttg
SBa_000599	crtI insulator	ccagtaacgttcatcagc
SBa_000601	dxs insulator	ggtcattacaacggttat
SBa_000607	vioB insulator	ccccagtcgagtcgtctata
SBa_000608	vioE insulator	ccactgtgtactcgttataacatctggcagtttagcgcatcgtccgcgctggaaggcgaccgcttcgtcggcttggtgctggatcaagacaat cagttcgaccagaccgaagcgcgcctggcgtcttggcttgaagagatcaaacgcaccgttctgtaataa
SBa_000590	terminator	gcgccgcagccggatctctccacggtacaggcatggtatctgcgccaccagacgcagtttatgcgcccggaacagcgtctgacccgccat ttactgctgacggtcgataacgaccgcgaagccgtgcaccagcggatcctcggcctgtatcggcaaatcaacgcctcgcgggacgctttc gcgccgctggcccagcgccattcccactgcccgagcgcgctggaagagggtcgtttaggctggattagccgtggcctgctctatccgcag ctcgagaccgcgctgttttcactggcggaaaacgcgctaagccttcccatcgccagcgaactgggctggcatcttttatggtgcgaagcgat tcgccccgccgcgcccatggagccgcagcaggcgctggagagcgcgcgcgattatctttggcagcagagccagcagcgccatcagcg cc
SBa_000591	terminator	agtggctggaacagatgatttcccgtcagccgggactgtgcgggtagcctcggcggctacccgttaacgcctacagcacggtgcgtttaat ctcctcaagccagctcgccagacgcgcttcggtctggtcgaactggttatcctgatccagcaccagcccaacaaagcggtcgccttccagc gccgaggacgcgctgaattcataaccctcatttggccagctgccaatcatctgcgcgccgcgcgcgctcagggcgtcgaacagcgggcg catcccgctgacgaagttgtccggatagcctctctgatcgccgaggccgaacagcgccacggttttcccttcaggctggcgtcgtcgagg ccgctgataaattcgctccatgactcgctttcgcatccggcctccagccccggcagctggccgtcgccgagcgtcggcgtgcccagcagc agcaccggataggccataaagtcgtccagcgtcgtgcggttaatgttgaccggggcatc
SBa_000508	Insulator	tgcagttttattctctgccagcactgtaataggcactaa
SBa_000561	P_{tac} Promoter and lacI cassette	caattcgcgctaacttacattaattgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgtgccagctgcattaatgaatcggccaa cgcgcggggagaggcggtttgcgtattgggcgccagggtggttttttcttttcaccagtgagacgggcaacagctgattgcccttcaccgcct ggccctgagagagttgcagcaagcggtccacgctggtttgccccagcaggcgaaaatcctgtttgatggtggttgacggcgggatataac atgagctgtcttcggtatcgtcgtatcccactaccgagatatccgcaccaacgcgcagcccggactcggtaatggcgcgcattgcgcccag cgccatctgatcgttggcaaccagcatcgcagtgggaacgatgccctcattcagcatttgcatggtttgttgaaaaccggacatggcactcca gtcgccttcccgttccgctatcggctgaatttgattgcgagtgagatatttatgccagccagccagacgcagacgcgccgagacagaactta atgggcccgctaacagcgcgatttgctggtgacccaatgcgaccagatgctccacgcccagtcgcgtaccgtcttcatgggagaaaataat actgttgatgggtgtctggtcagagacatcaagaaataacgccggaacattagtgcaggcagcttccacagcaatggcatcctggtcatcca gcggatagttaatgatcagcccactgacgcgttgcgcgagaagattgtgcaccgccgctttacaggcttcgacgccgcttcgttctaccatc gacaccaccacgctggcacccagttgatcggcgcgagatttaatcgccgcgacaatttgcgacggcgcgtgcagggccagactggaggt ggcaacgccaatcagcaacgactgtttgcccgccagttgttgtgccacgcggttgggaatgtaattcagctccgccatcgccgcttccacttt ttcccgcgttttcgcagaaacgtggctggcctggttcaccacgcgggaaacggtctgataagagacaccggcatactctgcgacatcgtata acgttactggtttcacattcaccaccctgaattgactctcttccgggcgctatcatgccataccgcgaaaggttttgcaccattcgatggtgtca acgtaaatgcatgccgcttcgccttcgcgcgcgaattggccgccatgccggcgataatggcctgcttctcgccgaaacgtttggtggcggg accagtgacgaaggcttgagcgagggcgtgcaagattccgaataccgcaagcgacaggccgatcatcgtcgcgctccagcgaaagcgg tcctcgccgaaaatgacccagagcgctgccggcacctgtcctacgagttgcatgataaagaagacagtcataagtgcggcgacgatagtc atgccccgcgcccaccggaaggagctgactgggttgaaggctctcaagggcatcggcggagcttatcgactgcacggtgcaccaatgctt ctggcgtcaggcagccatcggaagctgtggtatggctgtgcaggtcgtaaatcactgcataattcgtgtcgctcaaggcgcactcccgttct ggataatgtttttgcgccgacatcataacggttctggcaaatattctgaaatgagctgttgacaattaatcatcggctcgtataatgtgtggaatt

		gtgagcggataacaatt
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