Supplemental Information for:

Linear DNA for rapid prototyping of synthetic biological circuits in an *Escherichia coli* based TX-TL cell-free system

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Included in this insert are Supplemental S1 – S3, Table S1 – S2, Figures S1 – S13, and Supplemental References.

Supplemental S1.

We required a method to accurately quantify broad ranges of DNA, and tested both spectrophotometry and fluorometry. Both have known advantages and disadvantages: in particular, spectrophotometry is known to be inaccurate at low DNA concentrations, while fluorometry can produce biased plasmid DNA results due to conformational changes.[1] Comparing spectrophotometry (Nanodrop 2000) to fluorometry (Qubit 2.0 dsDNA HS and BR Assay), we found that linear DNA and plasmid DNA were most accurate and precise on the Nanodrop when at concentrations above 30 ng/μL, incurring at most 5.17% error (Figure S1, Table S1). However, for linear DNA from 2-30 ng/μL both of the dsDNA HS and BR Assays had superior accuracy and precision, incurring at most 12.02% error. For plasmid DNA from 2-30 ng/μL only the dsDNA BR Assay using a linear standard was accurate and precise, incurring 5.07% error. Based on these results, for subsequent data we quantified all constructs above 30 ng/μL on the Nanodrop, end-working concentration linear DNA from 2-30 ng/μL using the Qubit dsDNA HS Assay, and end-working concentration plasmid DNA from 2-30 ng/μL either from diluted Nanodrop stocks above 30 ng/μL or using the dsDNA BR Assay.

DNA Quantification Materials and Methods

A Nanodrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific) and a Qubit 2.0 fluorometer (Invitrogen) were used to measure dsDNA concentration. Per run, either 500 ng/μL of 1 kb DNA ladder (New England Biolabs) or 500 ng/μL of supercoiled DNA ladder (New England Biolabs) were diluted 1:2 down to 0.98 ng/μL in TE buffer and used as experimental samples. For the Nanodrop, 2 μL of sample was used to determine concentration. For the Qubit, 2 μL of sample was combined with 198 μL of supplied reagent:buffer to determine concentration. Different standards were tested for the Qubit, depending on the assay (dsDNA BR or dsDNA HS) and the type of DNA quantified (linear or plasmid). Linear standards were supplied by the manufacturer; plasmid standards consisted of pUC19 vector at 1000 ng/μL (New England Biolabs) diluted 1:10 in TE for the dsDNA BR assay or 1:100 for the dsDNA HS assay.

Supplemental S2.

The following plasmids, relevant DNA pieces, and primers were used in the study, along with Addgene Plasmid Depository Information and sequence data (if applicable).

Plasmids

Name	Short ID	Addgene	Notes
pBEST-p15A-Pl-tetO1-UTR1-lacI-T500	1	45784	
pBEST-p15A-Pl-tetO1-UTR1-deGFP-T500	2	45392	
pBEST-p15A-Pl-lacO1-UTR1-TetR-T500	3		Derived from 2 with replacement of "deGFP" with "tetR" coding sequence
pBEST_OR2-OR1-Pr_UTR1_deCFP_T500	18		Derived from 21 with replacement of "deGFP" with "deCFP" coding sequence
pBEST_OR2-OR1-Pr_UTR1_deGFP-T500	21	40019	
pBADmod1-linker2-gamS	22	45833	
pBEST_OR2-OR1-Pr-UTR1_ClpX-T500	48		Derived from 21 with "ClpX" coding sequence from Genbank U00096.3 substituted for "deGFP" – used as T500 template for 134-137.
pBEST-colE1-Pl-tetO1-UTR1-deGFP-T500	58		Derived from 21 with replacement of "OR2-OR1-Pr" with "Pl-tetO1"
pBEST-2kblhr2-OR2-OR1-Pr_UTR1_deGFP_T500-1gltB2kb	87		Derived from gltB and lhr genes from Genbank U00096.3 cloned into 21 - directly after T500 from <3354960 (AAAACTA…):3356959 (…GTGCTTC)> and directly before OR2-OR1-Pr from <1731632(ACGGTGG…):1733631(…GGTCCGG). G->A silent mutation in T500
pBEST_OR2-OR1-Pr_UTR1-deGFP-T500 BsaI, BbsI-safe	105		Derived from 21 with silent mutations to make BsaI, BbsI compatible
pBEST-Pl-tetO1-tetR-linker-deGFP-T500	109		Post cloned 4-piece from Isothermal/Golden Gate assembly (GGA), using sequences from 2 (Pl-tetO1-UTR1), 3 (tetR+linker from primer extension), 21 (linker from primer extension + deGFP), and 105 (vector backbone).
pBEST-p15A-Pl-tetO1-UTR1-lacI-T500 BsaI, BbsI-safe	113		Derived from 1 with silent mutations to make BsaI, BbsI compatible
pBEST-p15A-OR2-OR1-Pr-UTR1-deGFP-T500	121		Derived from 1 with promoter replacement
pBEST-p15A-Pl-tetO1-deGFP-T500	122		"
pBEST-p15A-Pl-lacO1-deGFP-T500	123		"

pBEST-p15A-J23113-deGFP-T500	124		“
pBEST-p15A-J23114-deGFP-T500	125		“
pBEST-p15A-J23116-deGFP-T500	126		“
pBEST-p15A-J23150-deGFP-T500	127		“
pBEST-p15A-J23106-deGFP-T500	128		“
pBEST-p15A-J23151-deGFP-T500	129		“
pBEST-p15A-J23100-deGFP-T500	130		“
pBEST-p15A-J23101-deGFP-T500	131		“
pBEST-p15A-J23102-deGFP-T500	132		“
pBEST-p15A-pNull-deGFP-T500	133		“
P3U2C7T2-v1-1 (Pl-lacO1-deGFP)	134		Post cloned 5-piece GGA using sequences from 105 (backbone), 3 (Pl-lacO1), 21 (UTR1), 21 (deGFP), and 48 (T500).
P4U2C8T2-v1-1 (Pl-tetO1-deCFP)	135		Post cloned 5-piece GGA using sequences from 105 (backbone), 2 (Pl-tetO1), 21 (UTR1), 18 (deCFP), and 48 (T500).
P3U2C5T2-v1-2 (Pl-lacO1-tetR)	136		Post cloned 5-piece GGA using sequences from 105 (backbone), 3 (Pl-lacO1), 21 (UTR1), 3 (tetR), and 48 (T500).
P4U2C6T2-v1-2 (Pl-tetO1-lacI)	137		Post cloned 5-piece GGA using sequences from 105 (backbone), 2 (Pl-tetO1), 21 (UTR1), 113 (lacI), and 48 (T500).

Promoters, Regulatory Elements, and Coding Sequences

Name	Sequence
OR2-OR1-Pr	TGAGCTAACACCGTGCGTGTTGACAATTTTACCTCTGGCGGTGATAATGGTTGCA
Pl-tetO1	TCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACA
Pl-lacO1	ATAAATGTGAGCGGATAACATTGACATTGTGAGCGGATAACAAGATACTGAGCACA
J23113	CTGATGGCTAGCTCAGTCCTAGGGATTATGCTAGC
J23114	TTTATGGCTAGCTCAGTCCTAGGTACAATGCTAGC
J23116	TTGACAGCTAGCTCAGTCCTAGGGACTATGCTAGC
J23150	TTTACGGCTAGCTCAGTCCTAGGTATTATGCTAGC
J23106	TTTACGGCTAGCTCAGTCCTAGGTATAGTGCTAGC
J23151	TTGATGGCTAGCTCAGTCCTAGGTACAATGCTAGC
J23100	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGC
J23101	TTTACAGCTAGCTCAGTCCTAGGTATTATGCTAGC
J23102	TTGACAGCTAGCTCAGTCCTAGGTACTGTGCTAGC
pNull	ATTCTGGGATTATACAGTAGTAATCACTAATTTAC
UTR1	AATAATTTTGTTTAACTTTAAGAAGGAGATATA
T500	CAAAGCCCGCCGAAAGGCGGGCTTTTCTGT
deGFP	ATGGAGCTTTTCACTGGCGTTGTTCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGA

	AGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAA GACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGA GCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCT GGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAA GAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAG CGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCC CGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAA AGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGC CGCCGGGATCTAA
deCFP	ATGGAGCTTTTCACTGGCGTTGTTCCCATCCTGGTCGAGCTGGACGGCGACG TAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCT ACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGC CCTGGCCCACCCTCGTGACCACCCTGACCTGGGGCGTGCAGTGCTTCAGCCG CTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGA AGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAA GACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGA GCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCT GGAGTACAACTACATCAGCCACAACGTCTATATCACCGCCGACAAGCAGAA GAACGGCATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCAG CGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCC CGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAA AGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGC CGCCGGGATCTAA
lacI_GGA_safe	ATGAAACCAGTAACGTTATACGATGTCGCAGAGTATGCCGGTGTCTCTTATC AGACCGTTTCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGAAAACGC GGGAAAAAGTGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAACCGCG TGGCACAACAACTGGCGGGCAAACAGTCGTTGCTGATTGGCGTTGCCACCT CCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTCGCGGCGATTAAATCTCG CGCCGATCAACTGGGTGCCAGCGTGGTGGTGTCGATGGTAGAACGAAGCGG CGTCGAAGCCTGTAAAGCGGCGGTGCACAATCTTCTCGCGCAACGCGTCAG TGGGCTGATCATTAACTATCCGCTGGATGACCAGGATGCCATTGCTGTGGAA GCTGCCTGCACTAATGTTCCGGCGTTATTTCTTGATGTCTCTGACCAGACAC CCATCAACAGTATTATTTTCTCCCATGAGGACGGTACGCGACTGGGCGTGGA GCATCTGGTCGCATTGGGTCACCAGCAAATCGCGCTGTTAGCGGGCCCATTA AGTTCTGTCTCGGCGCGTCTGCGTCTGGCTGGCTGGCATAAATATCTCACTC GCAATCAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGT CCGGTTTTCAACAAACCATGCAAATGCTGAATGAGGGCATCGTTCCCACTGC GATGCTGGTTGCCAACGATCAGATGGCGCTGGGCGCAATGCGCGCCATTAC CGAGTCCGGGCTGCGCGTTGGTGCGGATATCTCGGTAGTGGGATACGACGA TACCGAGGACAGCTCATGTTATATCCCGCCGTTAACCACCATCAAACAGGAT TTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGG GCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAA AAACCACCCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCG ATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGT GA
tetR	ATGTCTAGATTAGATAAAAGTAAAGTGATTAACAGCGCATTAGAGCTGCTT AATGAGGTCGGAATCGAAGGTTTAACAACCCGTAAACTCGCCCAGAAGCTA GGTGTAGAGCAGCCTACATTGTATTGGCATGTAAAAAATAAGCGGGCTTTG CTCGACGCCTTAGCCATTGAGATGTTAGATAGGCACCATACTCACTTTTGCC CTTTAGAAGGGGAAAGCTGGCAAGATTTTTTACGTAATAACGCTAAAAGTTT TAGATGTGCTTTACTAAGTCATCGCGATGGAGCAAAAGTACATTTAGGTACA CGGCCTACAGAAAAACAGTATGAAACTCTCGAAAATCAATTAGCCTTTTTAT GCCAACAAGGTTTTTCACTAGAGAATGCATTATATGCACTCAGCGCTGTGGG GCATTTTACTTTAGGTTGCGTATTGGAAGATCAAGAGCATCAAGTCGCTAAA GAAGAAAGGGAAACACCTACTACTGATAGTATGCCGCCATTATTACGACAA GCTATCGAATTATTTGATCACCAAGGTGCAGAGCCAGCCTTCTTATTCGGCC

	TTGAATTGATCATATGCGGATTAGAAAAACAACTTAAATGTGAAAGTGGGTCTTAA
linker sequence for tetR-deGFP fusion	GGTGAAAACCTGTACTTCCAGTCTGGTGGTGCT

Primers to make linear sequences and other plasmids

CHA-R	TTTTATCTAATCTAGACATGTGGTATATCTC CTTCTTAAAGTTAA	Isothermal assembly piece 1 to make 109, 2 w/ ZS30432f
CHB	TTTAAGAAGGAGATATACCACATGTCTAGA TTAGATAAAAGTAAAGTGAT	Isothermal assembly piece 2 to make 109, 3 w/ CHB-R
CHB-R	ACCAGACTGGAAGTACAGGTTTTCACCAGA CCCACTTTCACATTTAAGT	Isothermal assembly piece 2 to make 109, 3 w/ CHB
CHC	AACCTGTACTTCCAGTCTGGTGGTGCTATGG AGCTTTTCACTGGC	Isothermal assembly piece 3 to make 109, 21 w/ CHC-R
CHC-R	CTTTGAGTGAGCTGATACCGCAGTCATAAG TGCGGCGA	Isothermal assembly piece 3 to make 109, 21 w/ CHC
CHD	CGTCGCCGCACTTATGACTGCGGTATCAGCT CACTCAAAG	Isothermal assembly piece 4 to make 109, 105 w/ ZS30432r
ZS3033f	TGAGCTAACACCGTGCGT	0 bp protection, 21 w/ ZS3033rb
ZS3033rb	ACAGAAAAGCCCGCCTTTCGGCGGGCTTTG CTCGAGTTAGATC	0 bp protection, 21 w/ ZS3033f
ZS3034f	CATGCTGAGCTAACACCG	5 bp protection, 21 w/ ZS3034ra
ZS3034ra	TCGACACAGAAAAGCCCG	5 bp protection, 21 w/ ZS3034f
ZS3035f	GTGTGTGCTGTTCCGCT	25 bp protection, 21 w/ ZS3035r
ZS3035r	AAGGCTCTCAAGGGCATC	25 bp protection, 21 w/ ZS3035f
ZS3036f	AAAACCGAATTTTGCTGG	100 bp protection, 21 w/ ZS3036r
ZS3036r	ATGATAAAGAAGACAGTCATAAGTGCG	100 bp protection, 21 w/ ZS3036f
ZS3037f	TGGCGAATCCTCTGACC	250 bp protection, 21 w/ ZS3037r or 121-133 w/ ZS30610r or 58, 134-137 w/ ZS3037r
ZS3037r	TCTTTCCTGCGTTATCCC	250 bp protection, 21 w/ ZS3037f or 134-137 w/ ZS3037f
ZS3038f	AAAGGGAATAAGGGCGACA	500 bp protection, 21 w/ ZS3038r
ZS3038r	AGCGCCACGCTTCCC	500 bp protection, 21 w/ ZS3038f
ZS30412f	TCCGGTGAGCTAACACC	0 bp protection, 87 w/ ZS303412r
ZS30412r	GTTTTACAGAAAAGCCCGC	0 bp protection, 87 w/ ZS303412f
ZS30413f	AGAAGTGAATGATCTACCGGTC	5 bp protection, 87 w/ ZS303413r
ZS30413r	AAGAGCATCCCGACAGC	5 bp protection, 87 w/ ZS303413f
ZS30414f	ATTACTCGCCCCAGAGGTT	25 bp protection, 87 w/ ZS303414r
ZS30414r	GACAAGGTTTCGCGTTG	25 bp protection, 87 w/ ZS303414r
ZS30415f	GTGGGGAAATCTTCTGCC	100 bp protection, 87 w/ ZS303415r
ZS30415r	CGGCGGGCGATAAAC	100 bp protection, 87 w/ ZS303415f
ZS30416f	GCTACGGCATCATCAGTC	250 bp protection, 87 w/ ZS303416r
ZS30416r	GGTGATGGTGTTGATTTCAC	250 bp protection, 87 w/ ZS303416f
ZS30417f	ACGGTGGCGAAATTCA	500 bp protection, 87 w/ ZS303417r
ZS30417r	GAAGCACAGGCCCACTAC	500 bp protection, 87 w/ ZS303417f
ZS30432f	ATGACTATCGCACCATCAGCTAACGATATC CGCCTGAT	Isothermal assembly piece 1 to make 109, 2 w/ CHA-R
ZS30432r	GCATCAGGCGGATATCGTTAGCTGATGGTG CGATAGTCA	Isothermal assembly piece 4 to make 109, 21 w/ CHD
ZS30433f	ATCTAGGTCTCTAACGATATCCGCCTGAT	GGA piece 1 to make 109, 2 w/ ZS30433r
ZS30433r	GTTATGGTCTCGACATGTGGTATATCTCCTT CTTAAAGTTAA	GGA piece 1 to make 109, 2 w/ ZS30433f
ZS30434f	GATACGGTCTCCATGTCTAGATTAGATAAA AGTAAAGTGAT	GGA piece 2 to make 109, 3 w/ ZS3081r

ZS30435r	GTGCCGGTCTCATACCGCAGTCATAAGTGC GGCGA	GGA piece 3 to make 109, 21 w/ ZS3081f
ZS30436f	GGTTTGGTCTCCGGTATCAGCTCACTCAAAG	GGA piece 4 to make 109, 105 w/ ZS30436r
ZS30436r	ACGTTGGTCTCTCGTTAGCTGATGGTGCGAT AGTC	GGA piece 4 to make 109, 105 w/ ZS30436f
ZS30512f	AACAGGGTCTCACATGGAGCTTTTCACTGG	GGA “C7”, “C8”, 21 w/ ZS30523r or 18 w/ ZS30523r
ZS30513r	GTCCGGGTCTCACGACTCTCAAGGGCATCG GT	GGA “T2”, 21 w/ ZS30524f
ZS30514f	GTCCTGGTCTCTATGCGTGGTTGTCTTCGTA CGTCCGTCACGTTC	GGA “v1-1”, 105 w/ ZS30514r
ZS30514r	ATATAGGTCTCTGTCGGGCATTGTCTTCGCT CCTTCCGGTGG	GGA “v1-1”, 105 w/ ZS30514f
ZS30515f	TAGCGGGTCTCTGTCGTGCCTTGTCTTCGTT ACGTCCGTCACGTTC	GGA “v1-2”, 105 w/ ZS30528r
ZS30521r	CGTAAGGTCTCAGCTTGCTGTGCTCAGTATC TCT	GGA “P4”, 2 w/ ZS3057f
ZS30522f	AGCCAGGTCTCAAAGCAATAATTTTGTTTA ACTT	GGA “U2”, 21 w/ ZS3059r
ZS30523r	TTAGTGGTCTCATTCATTAGATCCCGGCGGC	GGA “C7”, “C8”, 21 w/ ZS30512f or 18 w/ ZS30512f
ZS30524f	GGCTCGGTCTCATGAAGCATCTGGTGAATA ACTCGAG	GGA “T2”, 21 w/ ZS30513r
ZS30528r	AGGTGGGTCTCTATGCTATGTTGTCTTCGCT CCTTCCGGTGG	GGA “v1-2”, 105 w/ ZS30515f
ZS30534r	CGTAAGGTCTCAGCTTGCTGTGCTCAGTATC TTGT	GGA “P3”, 3 w/ ZS3057f
ZS3057f	AGAACGGTCTCAGCATTGCTGTTCCGCTGG	GGA “P3”, “P4”, 3 w/ ZS30534r or 2 w/ ZS30521r
ZS3059r	TCCCCGGTCTCACATGGTATATCTCCTTCTT A	GGA “U2”, 21 w/ ZS30522f
ZS30610r	GAAGATCATCTTATTAATCAGATAAAATAT	250 bp protection, 121-133 w/ ZS3037f
ZS30611f	ACGAGGCCCTTTCGTCT	250 bp protection, 109 w/ ZS30611r
ZS30611r	ACGAGGCCCTTTCGTCT	250 bp protection, 109 w/ ZS30611f
ZS3064f	T*G*AGCTAACACCGTGCGT	0 bp protection, 2 TS, 21 w/ ZS3064r
ZS3064r	A*C*AGAAAAGCCCGCCTTTCGGCGGGCTTT GCTCGAGTTAGATC	0 bp protection, 2 TS, 21 w/ ZS3064f
ZS3065f	T*G*A*G*C*TAACACCGTGCGT	0 bp protection, 5 TS, 21 w/ ZS3065r
ZS3065r	A*C*A*G*A*AAAGCCCGCCTTTCGGCGGGC TTTGCTCGAGTTAGATC	0 bp protection, 5 TS, 21 w/ ZS3065f
ZS3066f	C*A*TGCTGAGCTAACACCG	5 bp protection, 2 TS, 21 w/ ZS3066r
ZS3066r	T*C*GACACAGAAAAGCCCGCCTTTCGGCGG GCTTTGCTCG	5 bp protection, 2 TS, 21 w/ ZS3066f
ZS3067f	C*A*T*G*C*TGAGCTAACACCG	5 bp protection, 5 TS, 21 w/ ZS3067r
ZS3067r	T*C*G*A*C*ACAGAAAAGCCCGCCTTTCGG CGGGCTTTGCTCG	5 bp protection, 5 TS, 21 w/ ZS3067f
ZS3068f	T*G*GCGAATCCTCTGACC	250 bp protection, 2 TS, 21 w/ ZS3068r
ZS3068r	T*C*TTTCCTGCGTTATCCC	250 bp protection, 2 TS, 21 w/ ZS3068f
ZS3069f	T*G*G*C*G*AATCCTCTGACC	250 bp protection, 5 TS, 21 w/ ZS3069r

ZS3069r	T*C*T*T*T*CCTGCGTTATCCC	250 bp protection, 5 TS, 21 w/ ZS3069f
ZS3071f	/5Alex594N/TGGCGAATCCTCTGACC	250 bp protection, 5' AF594, 21 w/ ZS3071r
ZS3071r	/5Alex594N/TCTTTCCTGCGTTATCCC	250 bp protection, 5' AF594, 21 w/ ZS3071f
ZS30810f	CAACCACGCATTGCTGTT	Overlap primers on "v1-1", 134-135 w/ ZS30810r
ZS30810r	CAATGCCCGACTCTCAAG	Overlap primers on "v1-1", 134-135 w/ ZS30810f
ZS30811f	CAAGGCACGACTCTCAAG	Overlap primers on "v1-2", 136-137 w/ ZS30811f
ZS30811r	ACAACATAGCATTGCTGTTC	Overlap primers on "v1-2", 136-137 w/ ZS30811r
ZS3081f	TCCTTGGTCTCGCTTCCAGTCTGGTGGTGCTATGGAGCTTTTCACTGGC	GGA piece 3 to make 109, 21 w/ ZS30435r
ZS3081r	TAACCGGTCTCAGAAGTACAGGTTTTCACCAGACCCACTTTCACATTTAAGT	GGA piece 2 to make 109, 3 w/ ZS30434f

Supplemental S3.

Inducible promoters Pl-tetO1 and Pl-lacO1 in linear and plasmid DNA were fit to a standard Hill function to approximate Michaelis-Menten dynamics using Prism 6.0 software (GraphPad Software, Inc.), which assumes a hill slope of 1.0:

$$[\boldsymbol{deGFP}] = \frac{\boldsymbol{V_{max}}[\boldsymbol{inducer}]}{\boldsymbol{K_m} + [\boldsymbol{inducer}]}$$

Table S1. Comparing absorbance and fluorometric quantifications of linear and plasmid DNA. (numerical). Data from Figure S1 in numerical form, with percent error from expected value included.

Linear	**Nanodrop**		**BR linear standard**		**HS linear standard**	
Expected (ng/μL)	Actual (ng/μL)	% Error	Actual (ng/μL)	% Error	Actual (ng/μL)	% Error
500	490.8 +/ 4.2	1.84%	471.7 +/- 41.3	5.67%		
250	251.3 +/- 2.1	0.53%	229.7 +/- 2.5	8.13%		
125	126.7 +/- 3.6	1.36%	114.7 +/- 1.2	8.27%	55	56.00%
62.5	63.80 +/- 3.10	2.08%	53.63 +/-2.24	10.99%	55.33 +/- 1.15	11.47%
31.25	32.87 +/- 2.61	5.17%	27.73 +/- 2.00	11.25%	28.07 +/- 2.38	10.19%
15.625	17.33 +/- 1.99	10.93%	13.97 +/- 1.40	10.61%	13.77 +/- 1.76	11.89%
7.8125	9.60 +/- 1.84	22.88%	6.97 +/- 0.92	10.74%	7.02 +/- 1.09	10.14%
3.90625	5.67 +/- 1.50	45.07%	3.51 +/- 0.82	10.06%	3.44 +/- 0.69	12.02%
1.953125	3.70 +/- 0.82	89.44%	1.77 +/- 0.54	9.38%	1.78 +/- 0.32	8.86%
0.976563	2.50 +/- 1.13	156.00%	1.17 +/- 0.05	19.30%	0.91 +/- 0.15	6.65%

Plasmid	**Nanodrop**		**BR linear standard**		**HS linear standard**		**BR plasmid standard**		**HS plasmid standard**	
Expected (ng/μL)	Actual (ng/μL)	% Error	Actual (ng/μL)	% Error	Actual (ng/μL)	% Error	Actual (ng/μL)	% Error	Actual (ng/μL)	% Error
500	494.2 +/- 7.1	1.15%	516.0 +/- 24.3	3.20%			556.7 +/- 47.3	11.33%		
250	255.7 +/- 4.6	2.28%	244.7 +/- 6.4	2.13%			260.7 +/- 18.0	4.27%		
125	128.9 +/- 5.3	3.15%	122.0 +/- 7.5	2.40%			124.0 +/- 3.5	0.80%		
62.5	64.87 +/- 4.02	3.79%	59.90 +/- 4.16	4.16%	46.43 +/- 6.93	25.71%	61.10 +/- 3.47	2.29%		
31.25	32.83 +/- 3.32	5.07%	29.43 +/- 3.40	5.81%	19.10 +/- 0.72	38.88%	29.37 +/- 1.01	6.03%	27.13 +/- 1.93	13.17%
15.625	16.83 +/- 2.50	7.73%	14.83 +/- 2.11	5.07%	8.57 +/- 0.85	45.13%	14.53 +/- 0.74	6.99%	12.77 +/- 1.50	18.29%
7.8125	8.67 +/- 2.23	10.93%	7.54 +/- 1.19	3.49%	4.02 +/- 0.28	48.50%	7.32 +/- 0.31	6.26%	5.70 +/- 0.75	27.04%
3.90625	4.57 +/- 1.70	16.91%	3.75 +/- 0.87	4.00%	1.99 +/- 0.20	49.14%	3.52 +/- 0.26	9.97%	2.90 +/- 0.22	25.76%
1.953125	2.80 +/- 1.73	43.36%	1.88 +/- 0.57	3.57%	0.99 +/- 0.06	49.33%	1.84 +/- 0.17	5.96%	1.44 +/- 0.07	26.27%
0.976563	1.60 +/- 1.40	63.84%	1.17 +/- 0.11	19.81%	0.50 +/- 0.04	48.77%	1.12	14.69%	0.72 +/- 0.08	26.48%

Table S2. Time Estimates of a Test Cycle in TX-TL. Time needed for rapid assembly in vitro versus traditional cloning, corresponding to Figure 6, is presented.

	Rapid Assembly[1]	Conventional Techniques (plasmid generation)
PCR of segments	na	1 h 15 min
DpnI digest[2, 3]	na	5 min
Assembly reaction[4]	1 h	1 h
Transformation and Recovery	na	1 h 30 min
Overnight growth on plates	na	16 h
Colony isolation and liquid media growth	na	8 h
Miniprep	na	30 min
PCR of rapid assembly product	1 h 15 min	
PCR Cleanup[3]	15 min	15 min
Setup TX-TL	15 min	15 min
TOTAL pre-TX-TL	2 h 45 min	1 d +
TOTAL post-TX-TL	4 h – 8 h	1 d +

[1] Rapid Assembly assumes the use of premade, re-usable modular parts – if these are not available, add 1 h 20 min to predicted time and follow beginning of "Conventional Techniques" protocol.

[2] Assumes the use of a fast-digest enzyme.

[3] During digest, run the previous reaction on an agarose gel to determine purity and reaction completion.

[4] Golden Gate Assembly has multiple protocols, from 1h to 3h20min in length. Protocol listed here assumes 10 cycles of 2min/37°C, 3min/20°C, 1 cycle 5min/50°C, 5min/80°C. Difficult assemblies can be accomplished by increasing cycling steps or by doing a constant at 37°C. Isothermal assembly can also be used in lieu.

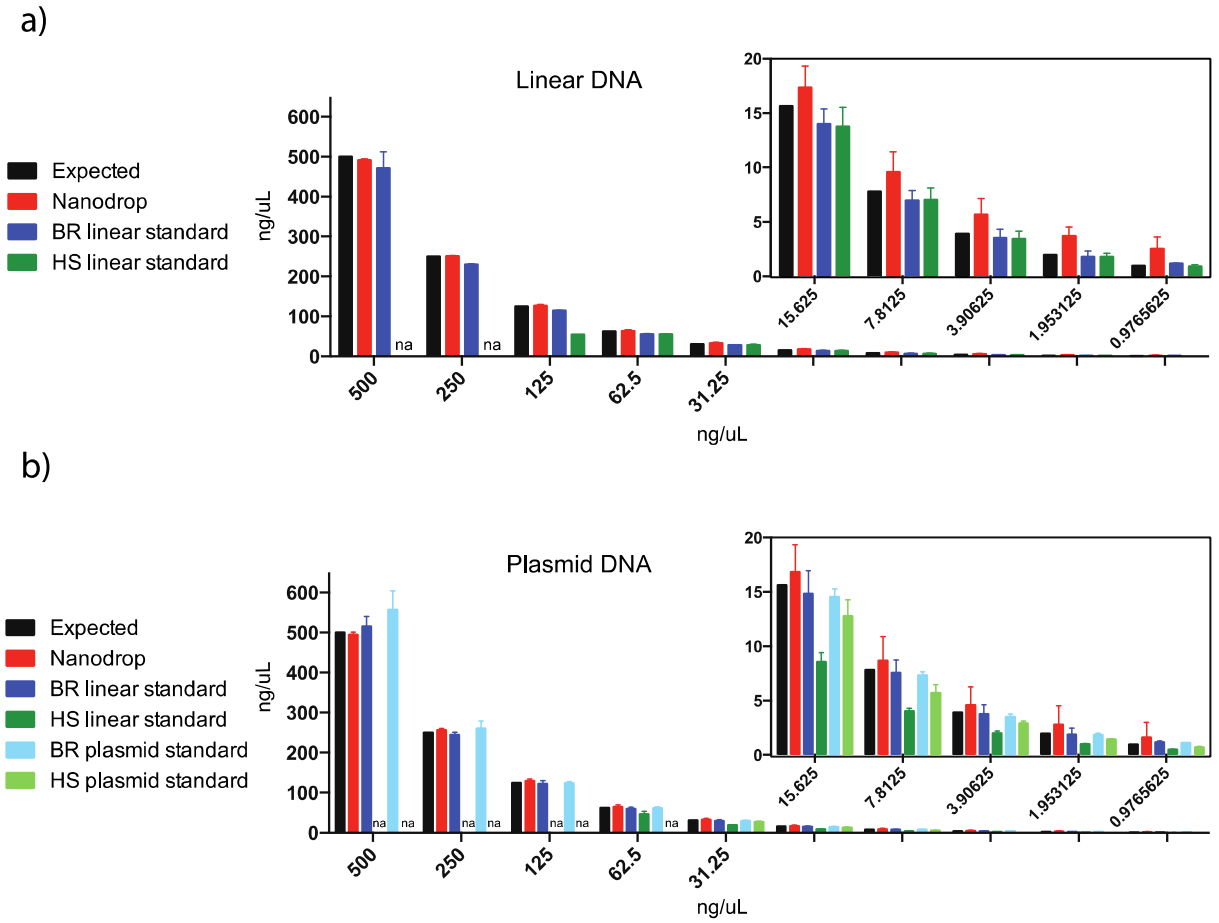

Figure S1. Comparing spectrophotomeric and fluorometric quantifications of linear and plasmid DNA. (graphic). a) 2 μL of 1kb linear ladder DNA at the expected ng/μL is either measured in the Nanodrop or the Qubit fluorometer using the dsDNA BR assay or dsDNA HS assay. Error bars represent a standard deviation from three independent samples, and "na" indicates out of range of the machine. b) Same process as a), but with supercoiled plasmid ladder DNA. BR linear standard: supplied with Qubit dsDNA BR Assay kit; HS linear standard: supplied with Qubit dsDNA HS Assay kit; BR plasmid standard: pUC19 plasmid DNA of known concentration at 0 ng/μL and 100 ng/μL in TE buffer; HS plasmid standard: pUC19 at 0 ng/μL and 10 ng/μL in TE buffer.

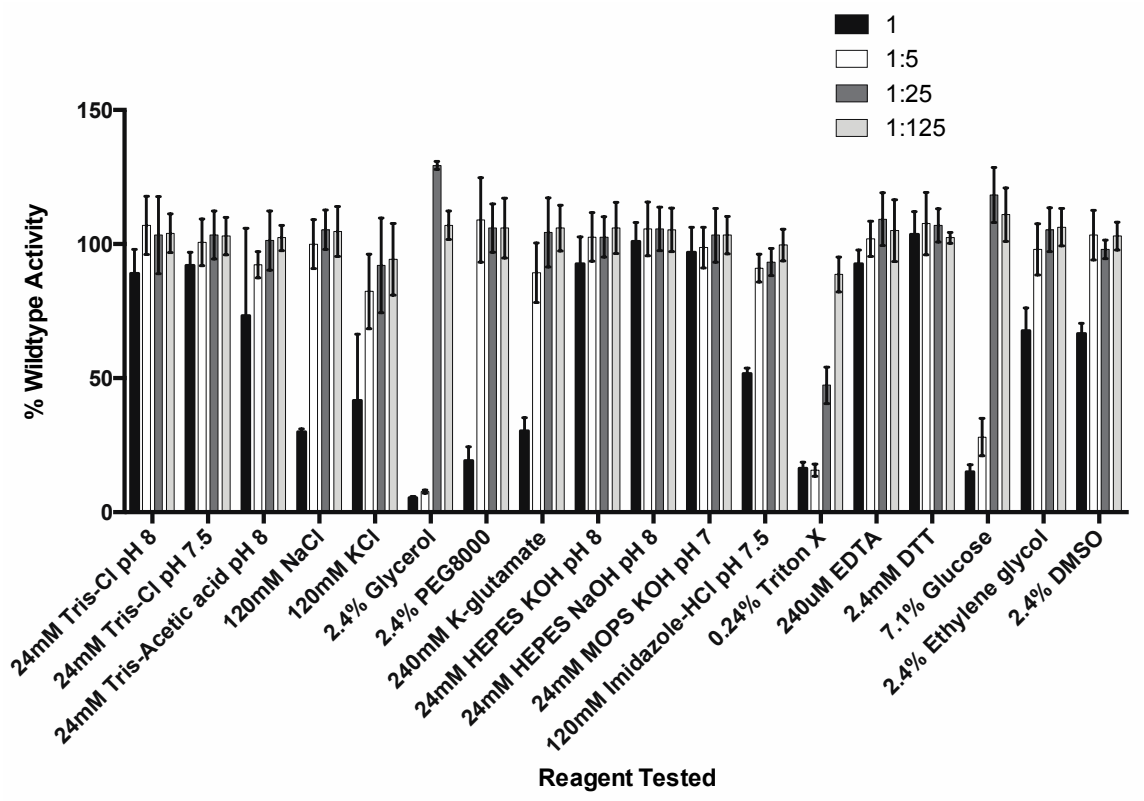

Figure S2. Effects of different additives on TX-TL efficiency. A variety of different additives commonly used in protein buffers are tested for toxicity. Endpoint fluorescence after 8 hours is determined for 1nM of pBEST-OR2-OR1-Pr-UTR1-deGFP-T500 at the final working concentrations listed in TX-TL or at 1:5 dilutions. Percent wildtype activity is against a control with no additive. Error bars represent one standard deviation from three independent experiments. Experiment is done in extract "e10."

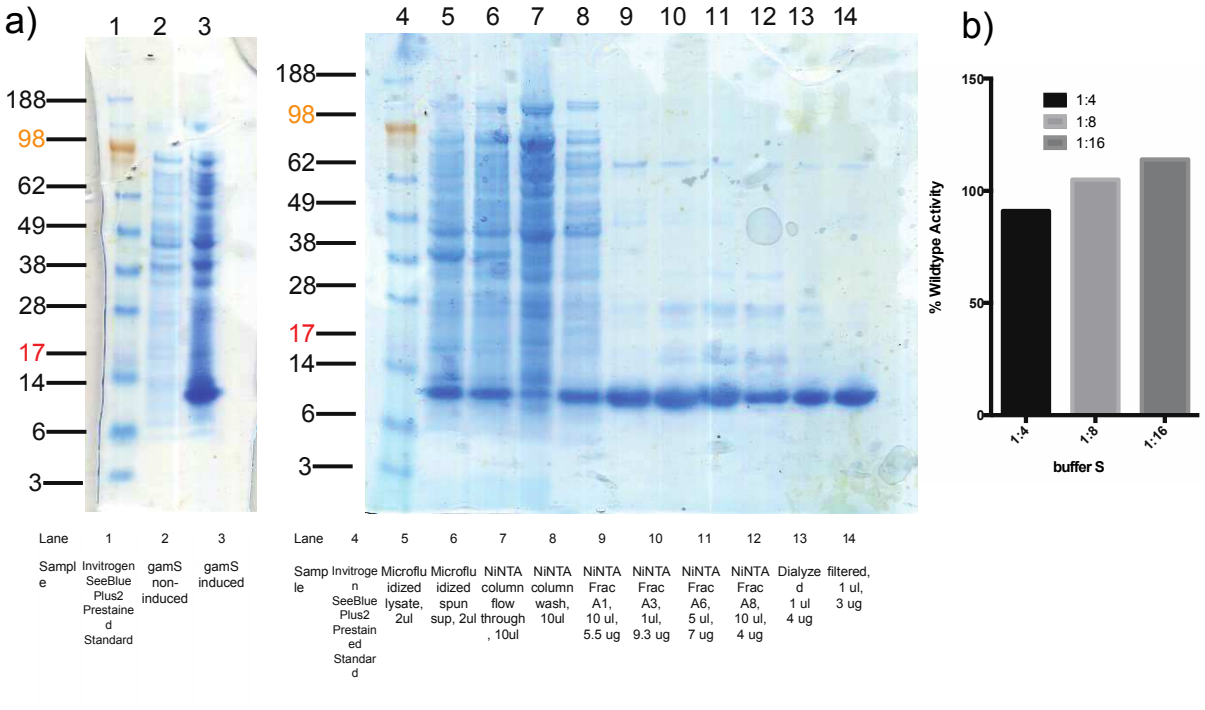

Figure S3. Purification of gamS protein into gamS storage buffer S. a) GamS protein is purified, expressed, and concentrated into 3 mg/ml as described in "Materials and Methods." Shown is the Coomassie Brilliant Blue stain of the purification procedure. b) Buffer toxicity of gamS storage buffer in TX-TL at different dilutions. Storage buffer composition ("buffer S") is 50 mM Tris-Cl pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 2% DMSO. Experiment is done in extract "e10."

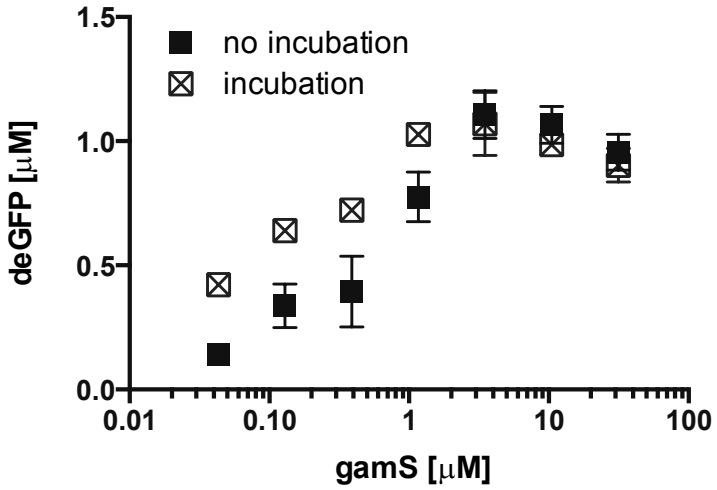

Figure S4. Effect of incubation time of gamS protein on linear protection. GamS protein to the listed concentration is added either directly to the crude cell extract for 30 minutes at room temperature ("incubation," square-x) or directly to the DNA ("no incubation," black-x). In the "incubation" case, crude cell extract incubated with gamS protein is then moved to 4°C and added to DNA. In the "no incubation" case, crude cell extract at 4°C is added directly to a mix of DNA and gamS protein. Reaction is run with 2 nM of linear DNA with no protection, and deGFP endpoint signal is measured. Error bars represent one standard deviation from three independent experiments.

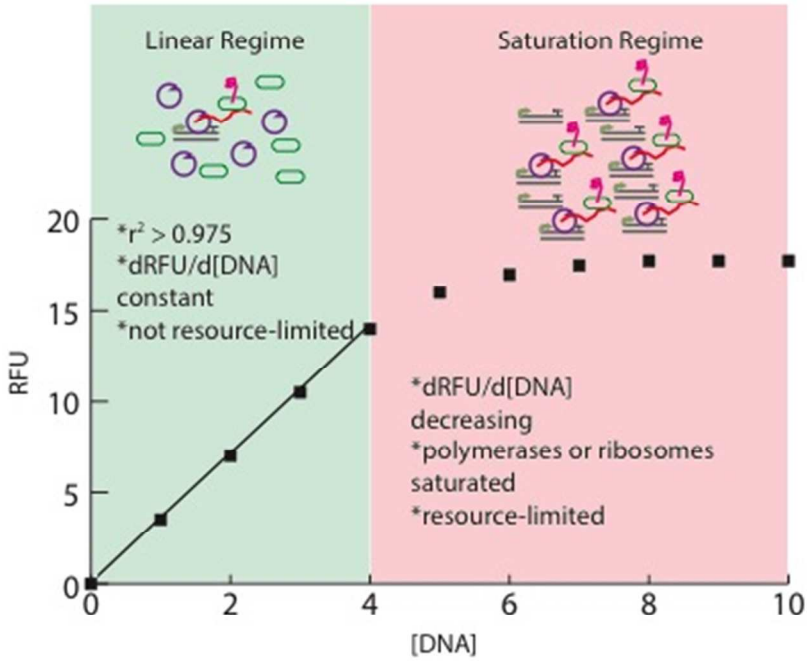

Figure S5. Definition of linear regime and saturation regime in TX-TL. Cartoon diagram shows hypothetical reaction with reporter protein, where rate of signal increase with DNA is constant up to 4 nM ("linear regime", green), begins to slow from 4 nM to 7 nM before becoming 0 above 7 nM ("saturation regime," red). The linear regime is not resource-limited, while the saturation regime is resource limited. Purple semicircle: RNA polymerase; green oval: ribosome; grey lines: DNA; red line: mRNA; pink squiggly: protein.

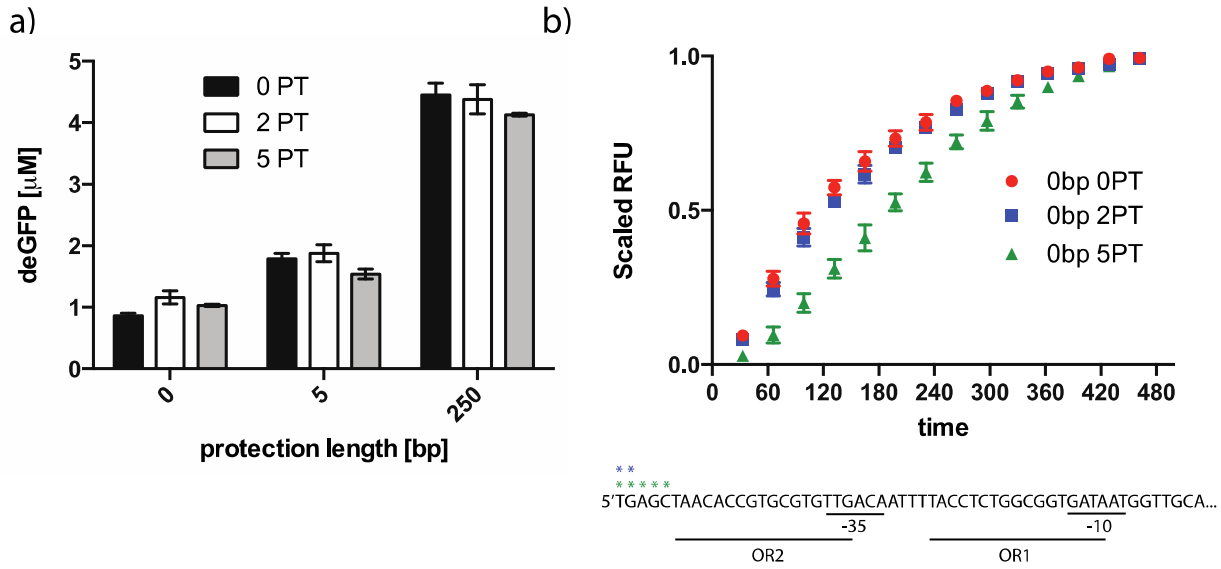

Figure S6. Protection of linear constructs with varying amounts of phosphorothioates. a) Endpoint expression of 2 nM linear DNA with 0, 2, or 5 phosphorothioates ("PT") on the 5" end on constructs with 0 bp, 5 bp, or 250 bp of non-coding DNA protection. b) Top, time-series data of expression from 2nM of a linear DNA construct with no non-coding sequence protection on either side of promoter OR2-OR-Pr and of terminator. All data series are scaled to an endpoint expression of 1.0 after 8 hours. Below, nucleotide sequence of the left side of the construct, with operator, promoter -35 and -10, and phosphorothioate sites notated.[2] Error bars represent one standard deviation from three independent experiments. Linear DNA is protected with gamS.

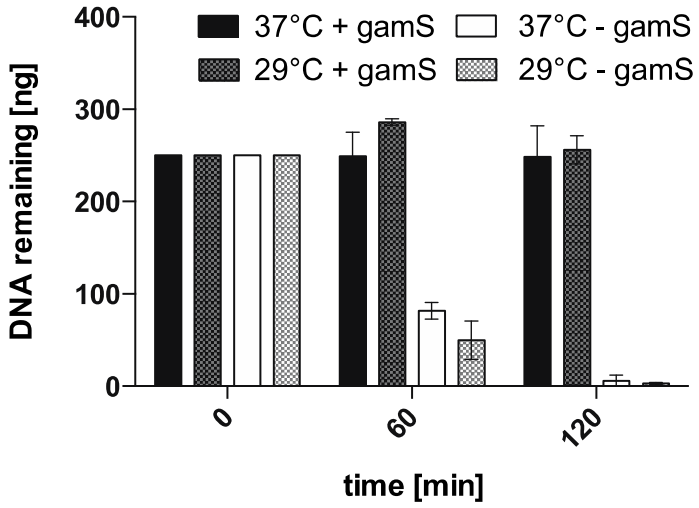

Figure S7. Degradation of saturating amounts of DNA in extracts prepared at different temperatures with and without gamS protein. Degradation rates of 250 ng (20 nM) of linear DNA with AlexaFluor-584 labeled dUTPs over time in extract produced at 37°C (extract "eZS1") or 29°C (extract "e13"). Signal is scaled to maximum DNA levels at time t=0. Error bars represent one standard deviation from three independent experiments.

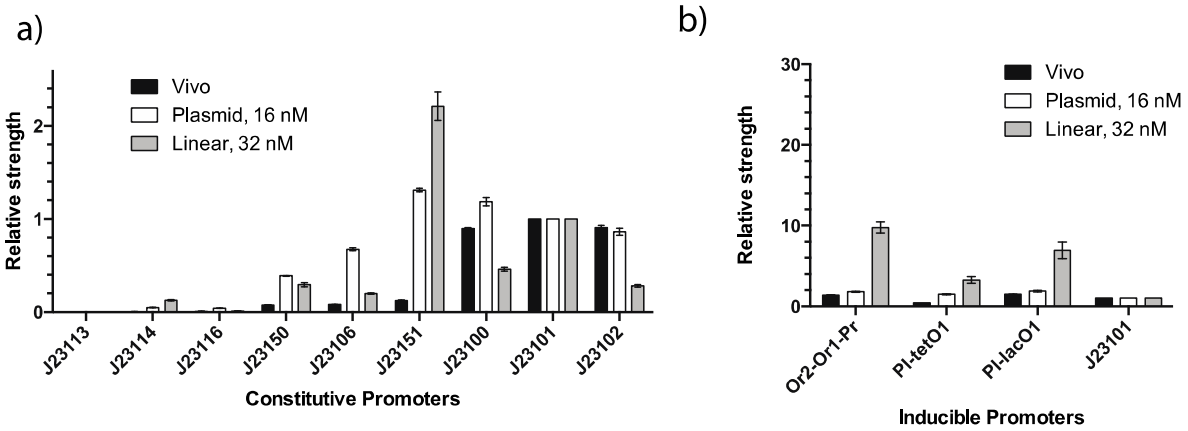

Figure S8: Comparison of strengths of different promoters using maximum measured amounts of DNA in TX-TL. a) Figure 4a is repeated for constitutive promoters but using maximum measured amounts of plasmid and linear DNA. b) Figure 4b is repeated for inducible promoters expressed constitutively.

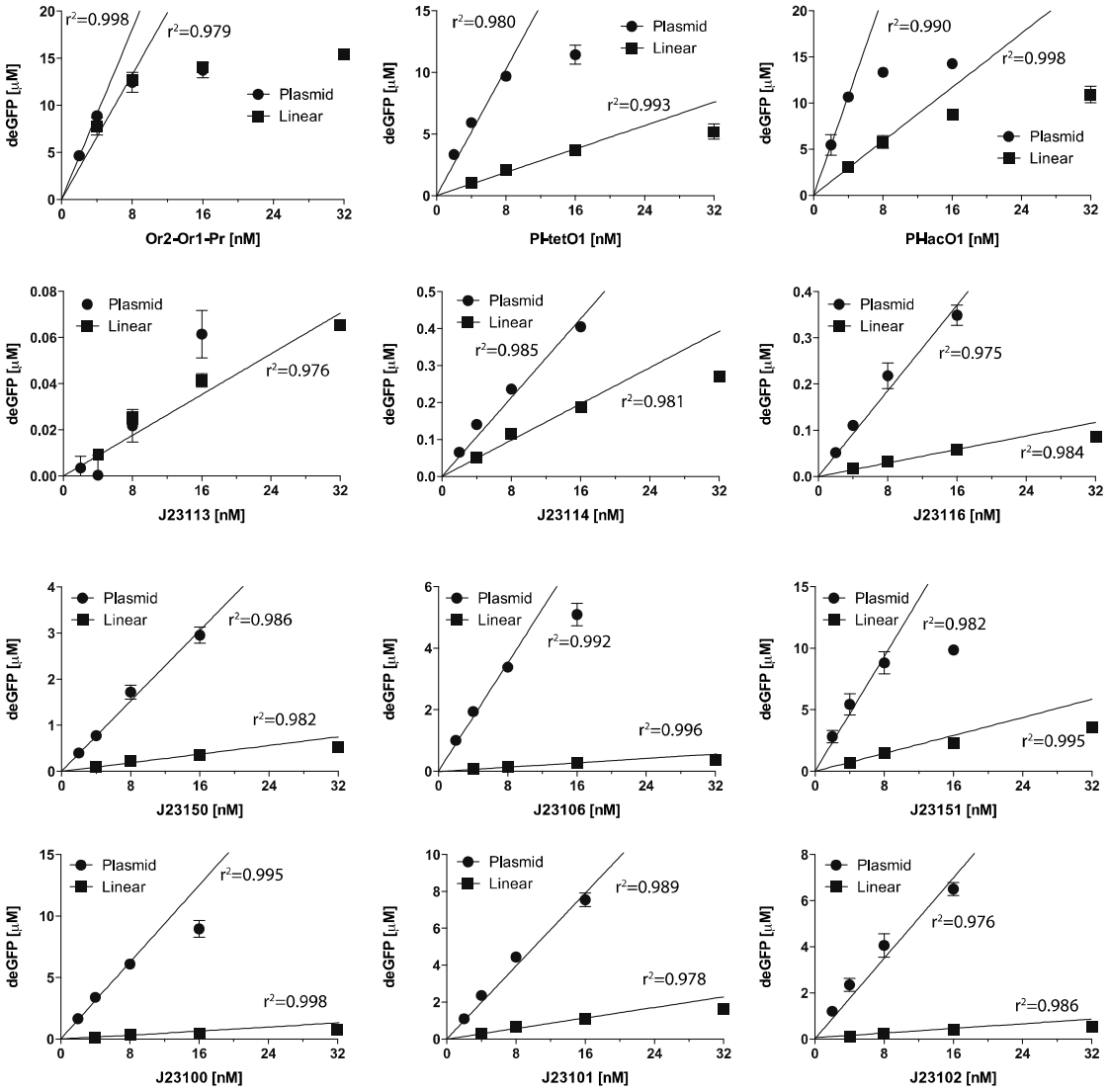

Figure S9. Individual traces of saturation curves. Saturation curves similar to Figure 4d, plotted for all promoters. r^2 and linear regression line are based on a cutoff of 0.975 and correspond to data from Table 1. Linear DNA is protected with 250bp of steric protection and with gamS. Error bars represent one standard deviation from three independent experiments.

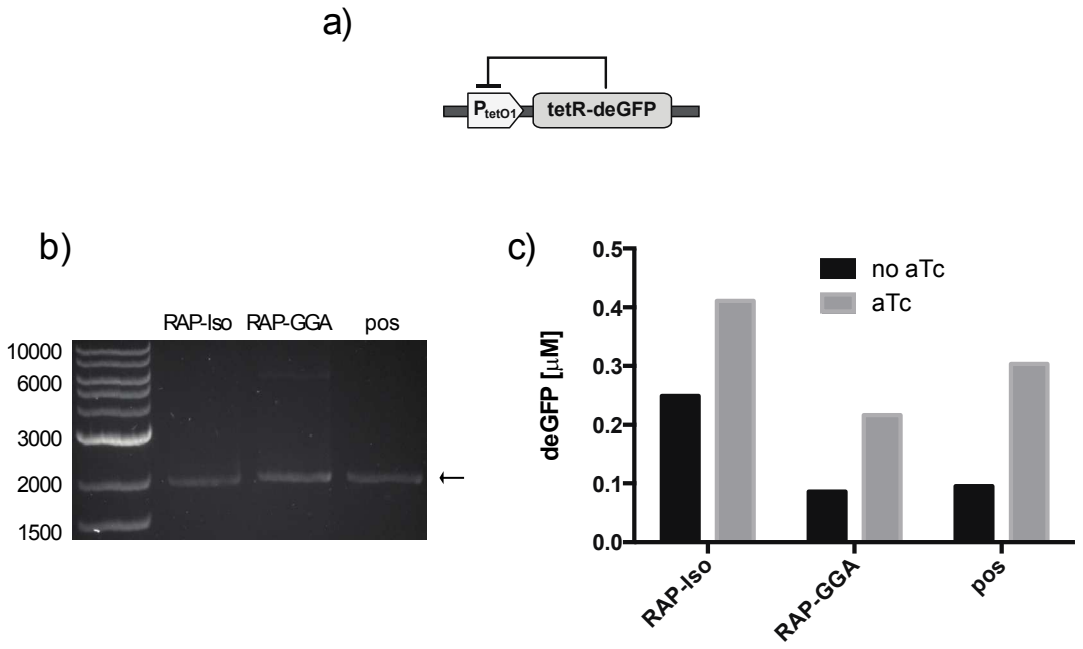

Figure S10. Rapid assembly and testing of a negative feedback gene. a) A four-piece negative feedback gene is assembled from standard pieces. b) Comparison by agarose gel electrophoresis of rapid assembly product made by Isothermal assembly ("RAP-iso"), rapid assembly product made by Golden Gate assembly ("RAP-GGA"), and post-cloned PCR product ("pos"). Arrow indicates expected band. Linear DNA is protected with 250 bp of steric protection and with gamS. c) Functional testing of 6 nM of rapid assembly products compared to post-cloned PCR product with or without 10 μM aTc.

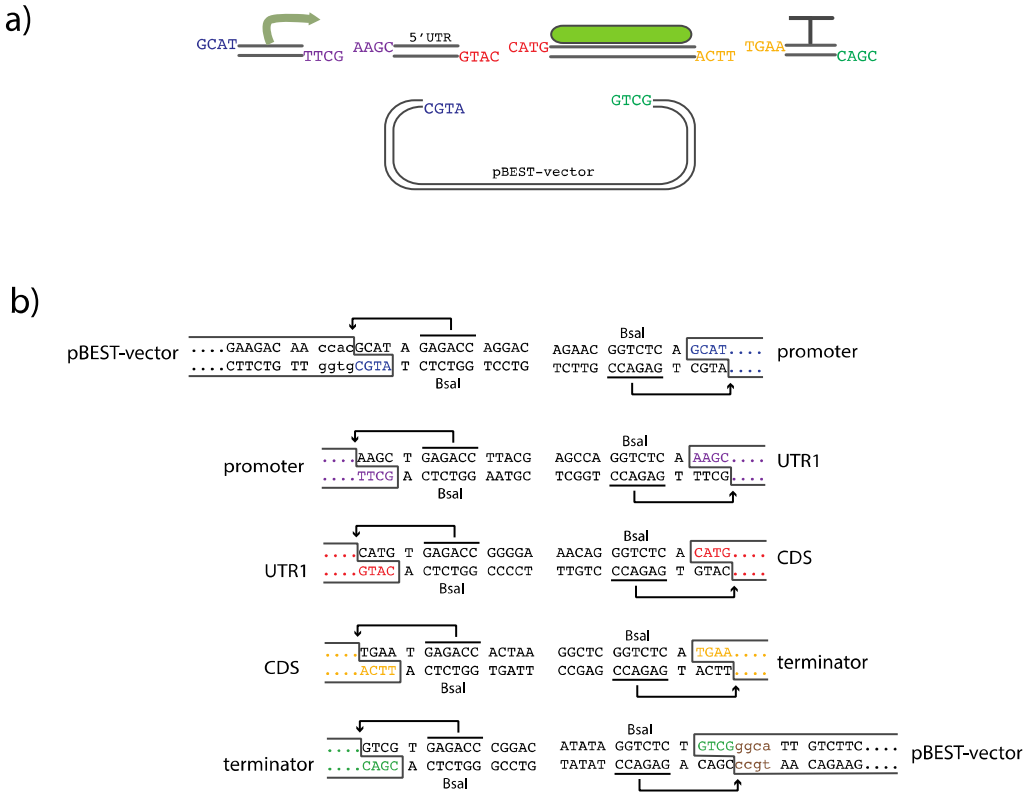

Figure S11. Overview of standard cloning procedure. a) A five-piece standard adopted with specific ligation ends for a promoter, 5' UTR, coding sequence, terminator, and vector based on the previously used pBEST backbone. b) Diagram of sequences for ligation at each site.

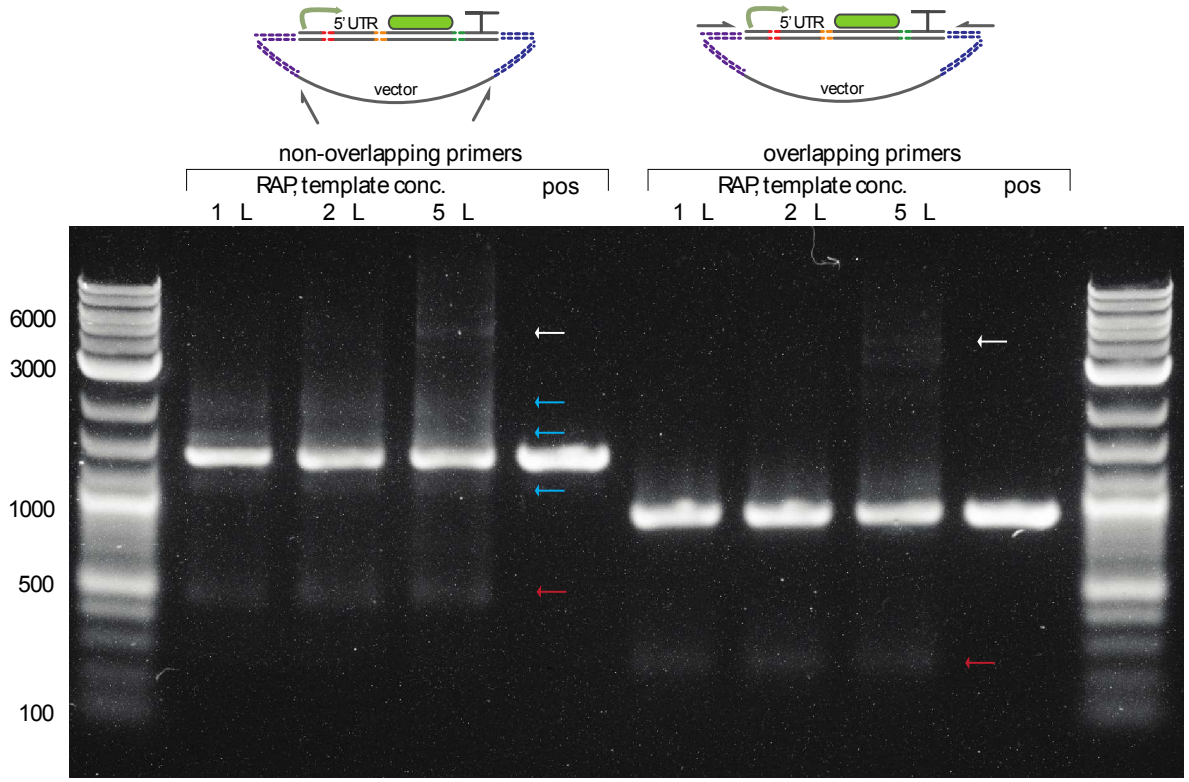

Figure S12. Purity of rapid assembly product as a function of template concentration and of overlapping primers. For a standard 5-piece assembly, the rapid assembly product ("RAP") is amplified off 1 μL, 2 μL, or 5 μL of template in a 50 μL PCR reaction. A post-cloned PCR product ("pos") is also produced. Non-overlapping primers refer to binding sites that do not cross the assembly junction between the vector and promoter and the vector and terminator; overlapping primers cross this junction. White arrow: template DNA; Blue arrows: Non-specific products removed by overlapping primers; Red arrow: non-specific products retained by overlapping primers. Red arrow is presumed to be self-ligated vector based on size.

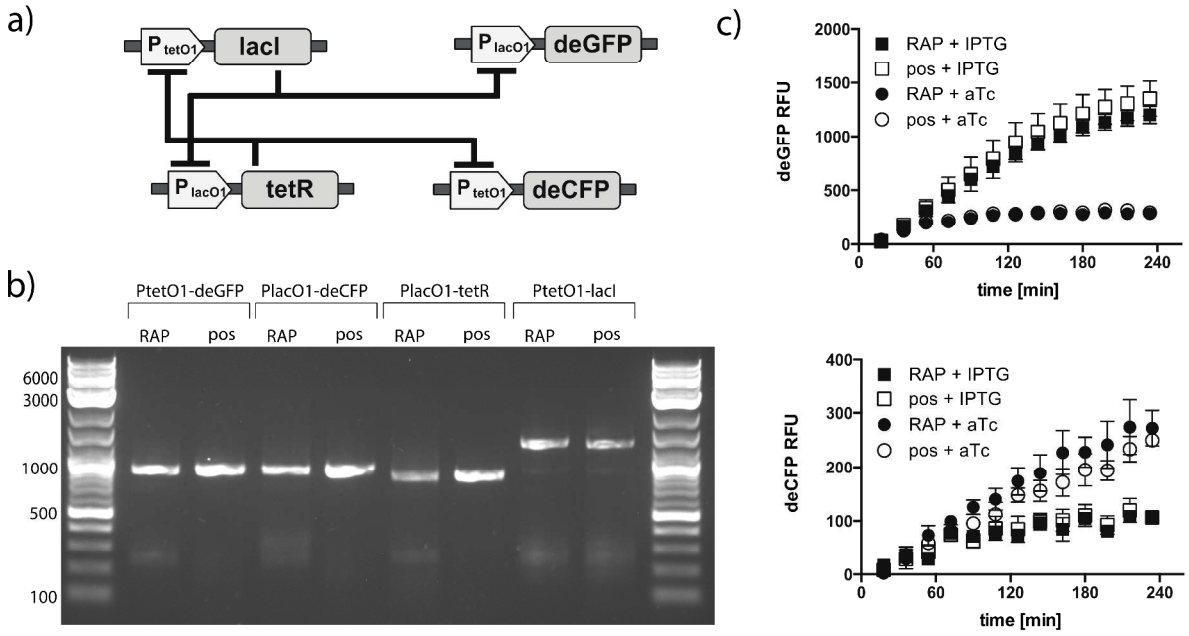

Figure S13: Rapid assembly of genetic switch. a) A four-piece genetic switch, identical to that in Figure 5a. b) Comparison of rapid assembly product ("RAP") to post-cloned PCR product ("pos") for four linear pieces formed, using overlap primers. c) Functional assay of RAP products versus post-cloned PCR products for "on" or "off" states of genetic switch. 2 nM of reporter and 1 nM of repressor is tested, and "+ IPTG" indicates the 0.5 mM IPTG, 0 μM aTc state while "- IPTG" indicates the 0 mM IPTG, 10 μM aTc state. Linear DNA is protected with 31 bp of steric protection and with gamS. Error bars represent one standard deviation from three independent experiments.

Supplementary Information References:

1. Labarca, C., and Paigen, K. (1980) A simple, rapid, and sensitive DNA assay procedure, *Analytical biochemistry 102*, 344-352.
2. Meyer, B. J., Maurer, R., and Ptashne, M. (1980) Gene regulation at the right operator (OR) of bacteriophage lambda. II. OR1, OR2, and OR3: their roles in mediating the effects of repressor and cro, *Journal of molecular biology 139*, 163-194.