Validation of an entirely *in vitro* approach for rapid prototyping of DNA regulatory elements for synthetic biology

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S1: Plasmid Description

Tables S1-S3 below describe all plasmids used in this study and the construction methods are described below each table. All plasmids in the table were newly constructed for this study. Oligonucleotides were ordered from Eurofins. Restriction digestions were performed with restriction enzymes from New England Biolabs (NEB). PCR was performed using *PfuUltra* II DNA polymerase from Agilent Genomics. Restriction digest products were purified directly or from agarose gel slice using Qiagen QIAquick PCR or Gel purification kits. For blunt end ligations, PCR products were phosphorylated using T4 Polynucleotide Kinase from NEB. Ligations were performed with T4 DNA ligase from NEB. Assembled plasmids were verified by restriction digestion and DNA sequencing.

Table S1: Minimal σ^{70} Constitutive Promoter Constructs

Promoter*	Sequence (5' to 3')	RBS*	Reporter*	Terminator*	Backbone*
J23100	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGC	B0034	GFPmut3b	B0015	pSB1A2
J23101	TTTACAGCTAGCTCAGTCCTAGGTATTATGCTAGC	B0034	GFPmut3b	B0015	pSB1A2
J23106	TTTACGGCTAGCTCAGTCCTAGGTATAGTGCTAGC	B0034	GFPmut3b	B0015	pSB1A2
J23113	CTGATGGCTAGCTCAGTCCTAGGGATTATGCTAGC	B0034	GFPmut3b	B0015	pSB1A2
J23114	TTTATGGCTAGCTCAGTCCTAGGTACAATGCTAGC	B0034	GFPmut3b	B0015	pSB1A2
J23116	TTGACAGCTAGCTCAGTCCTAGGGACTATGCTAGC	B0034	GFPmut3b	B0015	pSB1A2
J23102	TTGACAGCTAGCTCAGTCCTAGGTACTGTGCTAGC	B0034	GFPmut3b	B0015	pSB1A2
J23150	TTTACGGCTAGCTCAGTCCTAGGTATTATGCTAGC	B0034	GFPmut3b	B0015	pSB1A2
J23151	TTGATGGCTAGCTCAGTCCTAGGTACAATGCTAGC	B0034	GFPmut3b	B0015	pSB1A2

*DNA sequences obtained from the iGEM Registry of Standard Biological Parts (partsregistry.org)

To build the minimal σ^{70} constitutive promoter constructs, two oligonucleotides were designed so that once annealed they form a complementary double stranded helix containing single-stranded overhangs that are complementary to that of the GFP expression vector digested with EcoRI and XbaI.

Table S2: RBS Constructs

RBS*	Sequence (5' to 3')	Promoter*	Reporter*	Terminator*	Backbone*
B0030	ATTAAAGAGGAGAAA	J23101	GFPmut3b	B0015	pSB1A2
B0031	TCACACAGGAAACC	J23101	GFPmut3b	B0015	pSB1A2
B0032	TCACACAGGAAAG	J23101	GFPmut3b	B0015	pSB1A2
B0033	TCACACAGGAC	J23101	GFPmut3b	B0015	pSB1A2
B0034	AAAGAGGAGAAA	J23101	GFPmut3b	B0015	pSB1A2

*DNA sequences obtained from the iGEM Registry of Standard Biological Parts (partsregistry.org)

To build the RBS constructs, inverse PCR was performed with oligonucleotides containing overhangs to introduce the RBS into newly synthesized PCR products. PCR products were purified, phosphorylated and blunt ligations were performed.

Table S3: Inducible promoter constructs

Promoter	Sequence (5' to 3')	RBS*	Reporter*	Terminator*	Backbone**
pLasR3	TCGCACGGGAGGAGCCAGGAACAGCCTCCC TGGCTCCTCCCGCGGCGGTCTCGGCGCAGG GCGGATAACCCTATCGCGGCTATCCGTCGCT CCAGCGCGATATCGGCGGACAGCCCCTGCG CAGGCAAAGCGAACGCGACGCTGCGCGAAA CGCCTCCTTCCCTCCTCCCAGGCTCCCCCTC CCCATCGCGCCATCGCTCCGTACGCCTGCGC CTTTCACAGGCGGGTGACGAGACTTCCAGGT AGGAAATCCCTTCAAGCGCAACCAGGCAAGG ACGCGGTTGCCCAAGCCGTTTCCGAAGGTGC CTCCTGAGCAGTTCAGATAGGTTGTCCGACCC CGAGTGGCACGGCAGAGTGCCAGGGTTTATC	B0034	GFPmut3b	B0015	pSB1A2:LasR

	TCATAGGGACTTGAGC				
pLasRV	GTGTGTTGATGAGGAGACCCTAATCAGTGAGCCGCAATA CAGTACTTCTCAAGGTTACACGTATTATAGCTACGGAAGC TTCTATCGGTGTCCAGTCTTGATGGTCAAGACCATCCACG TTTTAGGTTGAACAAAGCAAAGCCTTTCCTTTAGGT	B0034	GFPmut3b	B0015	pSB1A2:LasR
pLasRL	GCGCGTCCCGGAGCTGGGGGCAACCTAGCTGCCACCTGCT TTTCTGCTAGCTATTCCAGCGAAAACATACAGATTTCCGGC GAAATCAAGGCTACCTGCCAGTTCTGGCAGGTTTGGCCGC GGGTTCTTTTTGGTACACGAAAGCACCGTCGAAAACGGGA CCGAGCCAGGGGAGTGCAGTTCCTTCTACCCGAAGGACTG ATACGGCTGTTCCGATCAGCCCACAAGGCGGCGGTAAGCG TCGGCCGAGTACTTCGGCCTGAAAAAACCAGGAGAACTGA ACAAG	B0034	GFPmut3b	B0015	pSB1A2:LasR

*DNA sequences obtained from the iGEM Registry of Standard Biological Parts (partsregistry.org)
**Vector backbone included the constitutive expression of LasR transcription factor using the regulatory elements: J23100:B0034:LasR(C0179):B0015

To build the LasR regulated promoter constructs, PCRs were performed on the *Pseudomonas aeruginosa* (PAO1) genomic DNA using standard protocols. Primers were designed to introduce EcoRI and SpeI sites into the PCR products. Following purification PCR products were digested with EcoRI and SpeI and ligated into the vector digested with EcoRI and XbaI.

S2. Primers used for production of linear DNA templates

Primers to generate linear PCR products for testing in cell-free systems were ordered from Eurofins MWG Operon. These PCR reactions were performed using *PfuUltra* II DNA polymerase from Agilent Genomics according to the manufacturer's protocol. Primers to generate the linear USER PCR products were ordered from Integrated DNA Technologies (IDT). These PCR reactions were performed with *PfuTurbo* C_x DNA polymerase from Agilent Genomics according to the manufacturer's protocol. The primers are shown below in Table S4.

Table S4: Primers used for production of linear DNA templates

Primer	Sequence (5' to 3')	Product size (bp)
Fwd linear	AGTGCCACCTGACGTCTAA	1155
Rev linear	ACCGCCTTTGAGTGAGCT	
Fwd Biotin	Biotin-AGTGCCACCTGACGTCTAA	1155
Rev Biotin	Biotin-ACCGCCTTTGAGTGAGCT	
Fwd Biotin Buffer	Biotin-GATAATACCGCGCCACATAG	1917
Rev Biotin Buffer	Biotin-GTTACCGGATAAGGCGCAG	
Fwd USER	AGUGCCACCUGACGUCUAAGAAACCATTATTATCATG	1118
Rev USER	ACCGCCTTTGAGUGAGCUGATACCGCTCGCCGC	

S3. Data analysis for characterization measurements in live *E.coli*

For the constitutive promoter and RBS library the steady-state fluorescence normalized to absorbance at 600 nm (cell number) was calculated from three independent repeats. For each repeat, each member was measured in triplicate and fluorescence (excitation at 485nm, emission at 520nm) and O.D.600 measured for 30 minutes at 5 minute intervals. In addition, *E. coli* containing the backbone vector and a GFP gene without a promoter (pSB1A2:GFPmut3b) and media only was measured in parallel.

For data analysis of the constitutive promoter library and the RBS library the background fluorescence of *E. coli* BL21-Gold (DE3) containing pSB1A2:GFPmut3b were removed from all fluorescent measurements. The background absorbance of M9 media only was removed from all absorbance measurements. For each member of the library, an average absorbance and fluorescence value was calculated from all the time points of the three triplicates. The relative strengths of promoter and RBS libraries were calculated as below:

$$\mathrm{Re}\,altive\_Strength(X) = \frac{Fl_X / ABS_X}{Fl_{Std} / ABS_{Std}}$$

where Fl_X and ABS_X is the fluorescence and O.D.600 of the promoter or RBS tested and Fl_{std} and ABS_{std} is the fluorescence and O.D.600 of the promoter (J23101) or RBS (B0034) that the libraries are normalized to. Finally the average and standard deviation of the three independent repeats were calculated to give final relative strengths.

For inducible promoters (pLasR) the steady-rate of fluorescence increase normalized to absorbance was calculated from three independent repeats. For each repeat, each member was measured in triplicate and fluorescence (excitation at 485nm, emission at 520nm) and O.D.600 measured for 6 hours at 5 minute intervals. In addition, *E. coli* containing pSB1A2:GFPmut3b and media only were measured in parallel.

As before, background fluorescence and absorbance were removed from *E. coli* containing vector without promoter and media only. The rate of fluorescence increase was calculated between 4 hours and 4 hours 15 minutes after induction, at which time it was shown that for each promoter a steady-rate of fluorescence increase was reached and maximal promoter activity was achieved as shown below:

$$Rate(X) = Fl_X t_{4hour} - Fl_X t_{4hour 15\,\mathrm{min}}$$

For each member of the library, an average absorbance and rate of fluorescence increase was calculated from all the three triplicates. The normalized relative strengths of inducible promoters libraries are calculated as below:

$$\mathrm{Re}\,altive\_Strength(X) = \frac{Rate_X / ABS_X}{Rate_{Std} / ABS_{Std}}$$

where Fl_X and ABS_X is the fluorescence and O.D.600 of the promoter tested and Fl_{std} and ABS_{std} is the fluorescence and O.D.600 of the promoter pLasR3 at 10 μM AHL that the library is normalized to. Finally the average and standard deviation of the three independent repeats were calculated to give final relative strengths.

S4. Data analysis for characterization measurements in *E.coli* extract cell-free system characterization

For the constitutive promoter, RBS and inducible promoter library three independent repeats were performed. For each repeat, the fluorescence of each member (excitation at 485nm, emission at 520nm) was measured for up to 4 hours at 15-minute intervals. In addition, a reaction containing the backbone vector with the GFP gene without a promoter (pSB1A2:GFPmut3b) was measured in parallel.

For data analysis the background fluorescence of a reaction containing pSB1A2:GFPmut3b was removed from all fluorescent measurements. For each member of the library, the rate of fluorescence increase was calculated between 30 minutes and 45 minutes of reaction initiation, as below:

$$Rate(X) = Fl_X t_{45\,\mathrm{min}} - Fl_X t_{30\,\mathrm{min}}$$

The rate is calculated between these measurements because it was shown that this is when promoter activity was at a steady-rate of expression and maximal within the cell-free systems

(Figure S1 and Figure S2). Next the relative strength of promoters and RBS is calculated as below:

$$\mathrm{Re}\,lative\_strength(X) = \frac{Rate_X}{Rate_{Std}}$$

where $Rate_X$ is the rate of fluorescence increase of each library member and $Rate_{std}$ is the rate of fluorescence increase of the σ^{70} promoter (J23101), RBS (B0034) or inducible promoter (pLasR3 at 10μM AHL) that the libraries are normalized to. Finally the average and standard deviation of the three independent repeats were calculated to give final relative strengths.

S5. Hill function fitting of inducible promoters in live *E. coli* and *E. coli* extract cell-free systems

For each inducible promoter in both *E.coli* and *E.coli* extract cell-free systems was fitted to a hill function equation as shown below, using curve fitting tool (cftool) in MATLAB from MathWorks.

$$\frac{\beta \bullet [inducer]^n}{Km^n + [inducer]^n}$$

The hill function equation approximates the does-response curve by the three parameters; β that defines the maximum transcription output and is determined directly from experimental data; *Km* (binding efficiency) that defines the induction threshold; and *n* (cooperativity) that defines the steepness of the curve.

Figure S1. Time course of the characterization data in cell-free systems. The (a) raw fluorescence and (b) the rate of fluorescence increase ($time_{15min}$-$time_{0min}$) over time of six σ^{70} promoters in *E. coli* extract cell-free systems.

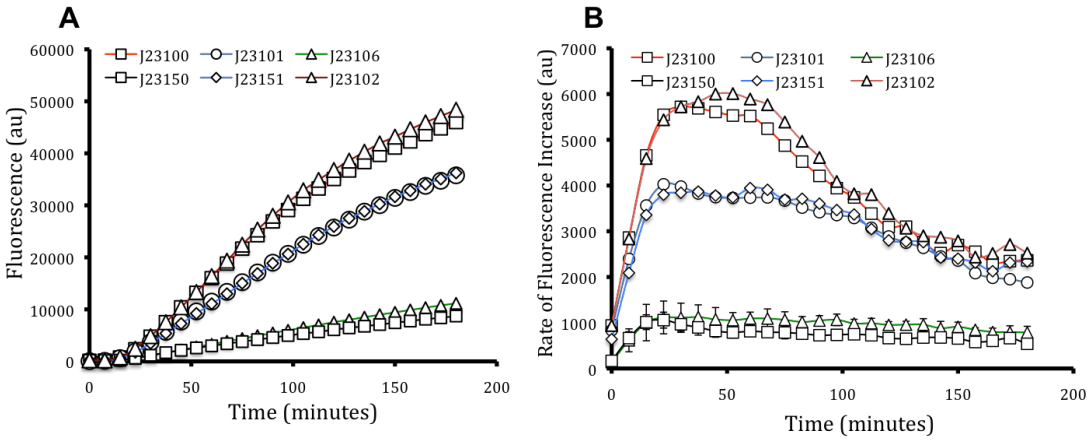


Figure S2 Examples of the rate of fluorescence increase ($time_{15min}$-$time_{0min}$) for the characterization of DNA regulatory elements in cell-free systems. (a) RBS, (b) pLasR promoters at 10 μM AHL, (c) constitutive promoters using linear DNA templates, (d) RBS using linear DNA templates and (e) constitutive promoters using USER-ligase DNA templates.

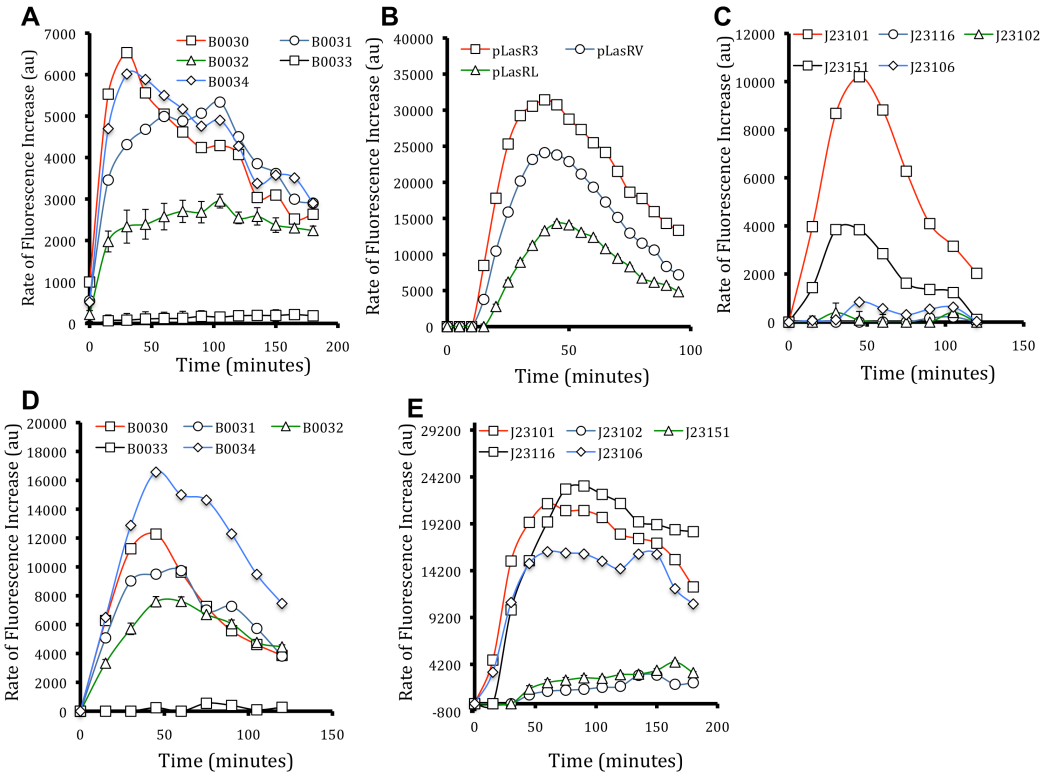


Figure S3. Comparison of the characterization of constitutive σ^{70} promoters collected *in vitro* and *in vivo*, with previously published data by Kelly et al (1).

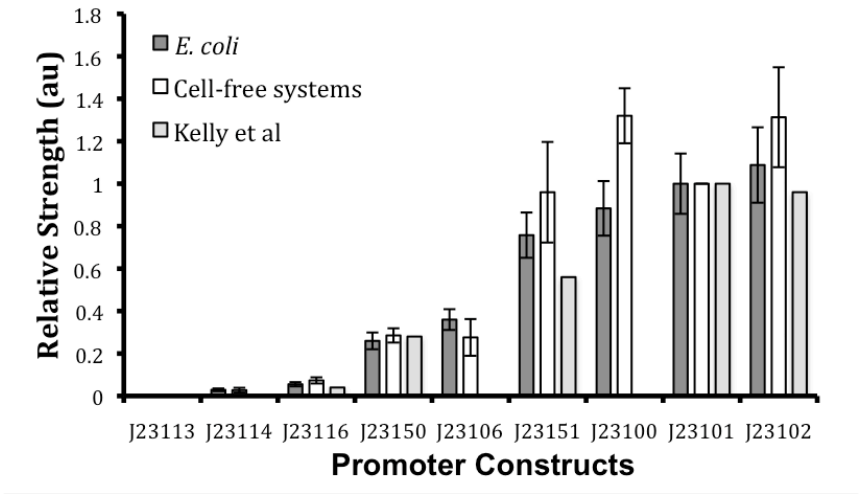


Figure S4. Correlation of characterization of constitutive σ^{70} promoters and RBS collected *in vitro* and *in vivo*. Linear regression analysis was performed on the relative strengths of (a) constitutive σ^{70} promoters and (b) RBS collected from live *E. coli* and cell-free systems.

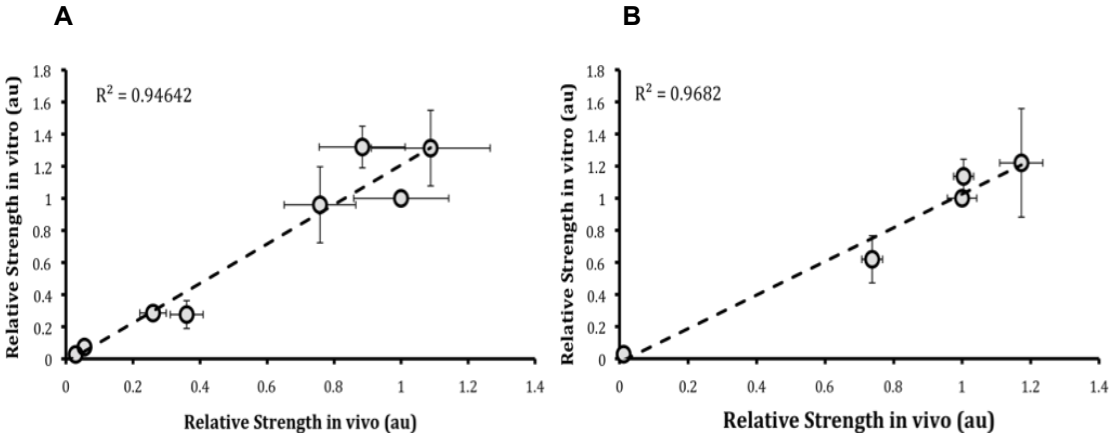


Figure S5. Comparison of linear DNA templates in cell-free systems. Comparison of a linear (1000 bp), 5' biotinylated linear (1155 bp) and 5' biotinylated linear including buffer region (1917 bp) were tested as DNA templates in *E. coli* extract and a modified *E.coli* linear extract cell-free systems. For experiments in cell-free systems 0.5 μg of linear and biotinylated linear (biotin linear) and 1 μg of biotinylated linear with a buffer region (biotin buffer linear) were added to each reaction and fluorescence measured at 30°C. Each template was from PCR of the J23101:B0034:GFP:B0015 construct. The relative strength was calculated from the rate of fluorescence increase in the maximal phase of GFP expression, 30 to 45 minutes after initiation and normalized to the strength of the biotinylated linear including a buffer region. The data represents the mean +/- the SD of three independent reactions.

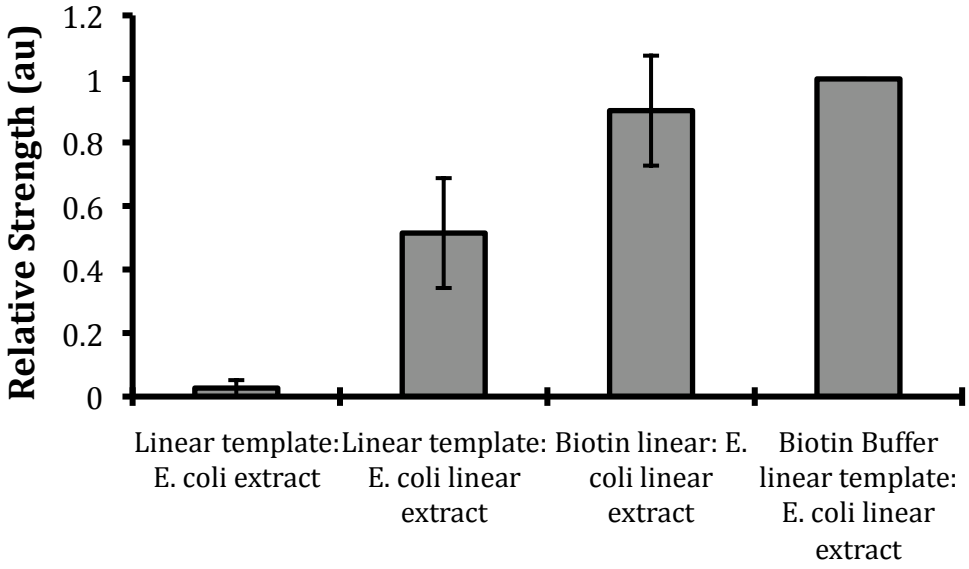


Figure S6. Products of a USER-ligase and USER-only reaction analysed on a GelRed stained agarose gel. L: 1kb plus DNA ladder; 1: PCR generated product; 2:USER-only products; and 3: USER-ligase products. Arrow indicates the closed-circular DNA. All lanes contain 300 ng of DNA.

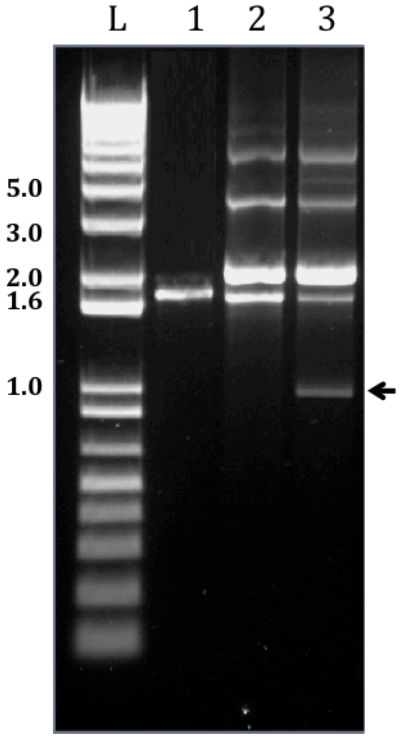
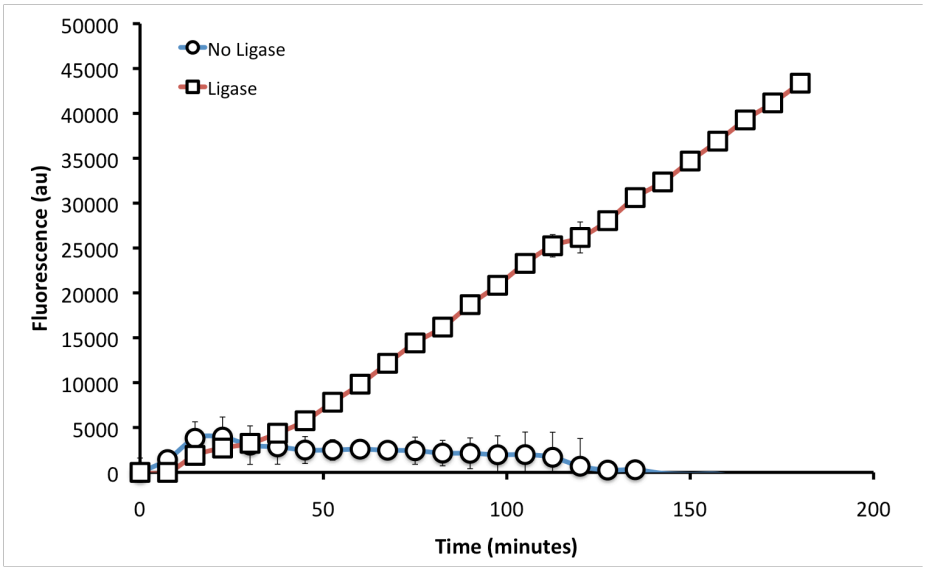


Figure S7. Comparison of the products of USER-ligase and USER-only reactions for DNA templates in cell-free systems. For experiments in cell-free systems 1 μg of DNA products from a USER-ligase and USER-only was added to each reaction and fluorescence measured at 30°C. The data represents the mean +/- the SD of three independent reactions.



SUPPLEMENTARY REFERENCES

1. Kelly JR, *et al.* (2009) Measuring the activity of BioBrick promoters using an in vivo reference standard. *J Biol Eng* 3:4.