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EMULSION BASED SELECTION OF T7 PROMOTERS OF VARYING ACTIVITY

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The ability to build and control complex biological systems is greatly enhanced by the generation of related parts with varying strengths. In this way, various parts can be strung together and the connectivity and expression levels can be matched for the desired system performance. Engineered gene circuits, both *in vivo* and *in vitro*, often utilize the T7 RNA polymerase in tandem with the T7 promoter for transcription. In this work, we describe the selection of T7 promoter variants of varying strength by emulsifying *in vitro* transcription with subsequent fluorescence activated cell sorting (FACS) to enrich for active promoters. Such variant promoters should be of use to synthetic biologists for both *in vivo* and *in vitro* applications.

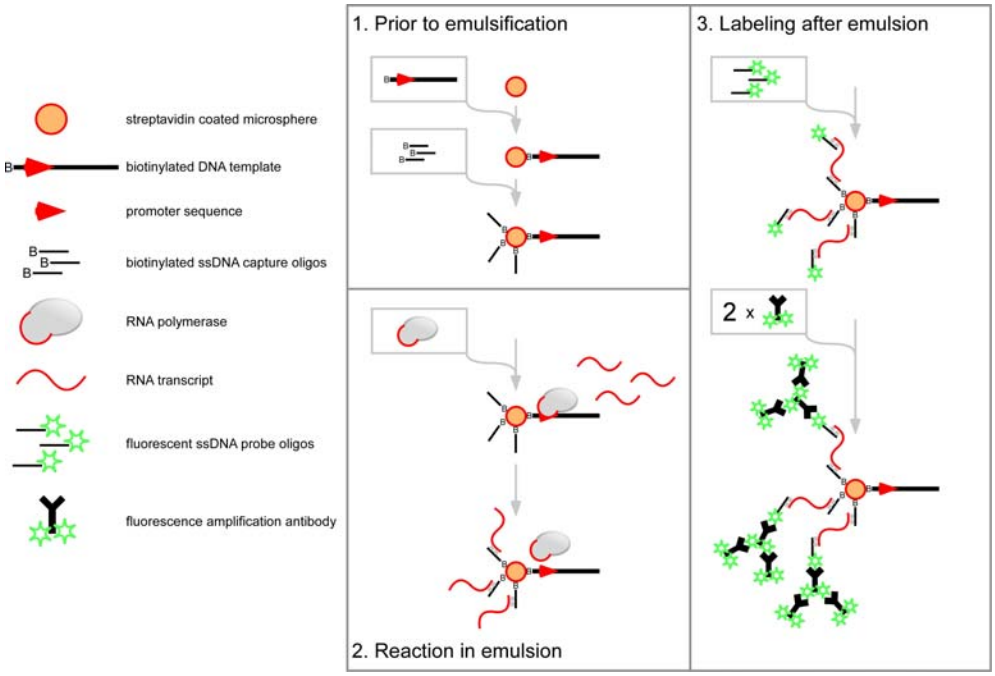
1. Introduction

T7 RNA polymerase (T7 RNAP) plays an important role in the production of RNA transcripts for biotechnology applications. For example, it is widely used for protein overproduction. It has also been routinely used for generating large quantities of functional RNAs *in vitro*, such as aptamers, ribozymes, and siRNAs. T7 RNAP has also played a key role in many engineered and synthetic genetic circuits[1-3], at least in part because a single, monomeric protein can act orthogonally on a short, 17 nt promoter[4]. Indeed, as scientists and engineers contemplate creation of an artificial, minimal cell, the T7 RNA polymerase is at the forefront of the discussion of how to power transcription[5].

Because of the difficulties inherent in modeling synthetic parts and circuits in organisms, the development of synthetic circuits in cell free settings, including artificial cell-like liposomes and water-in-oil emulsions, is an attractive alternative[6-8]. Indeed, cell-free systems are already being used for biomolecular engineering in order to bypass the systemic and evolutionary constraints inherent in living cells[6,9-12]. The shift from *in vivo* to *in vitro* circuitry may be particularly desirable for T7 RNA polymerase and its promoter, since in cells it is active to the point of toxicity when not exquisitely controlled (for example, see reference 13). Typically, synthetic biologists have chosen to control T7 driven gene expression by controlling the production of the T7 RNA polymerase[1].

However, just as the performance of parts *in vitro* is not necessarily predictive of their role *in vivo*, it will be necessary to determine how well individual parts function in emulsions and other cell-like environments. To generate parts for synthetic circuits and to better understand how such circuits can themselves adapt and evolve, we randomized the initially transcribed sequence (ITS) of the T7 promoter, which modulates transcription levels from the core phi10 T7 promoter, and selected for different T7 RNAP promoters with different transcription strengths in emulsions. These and additional selected promoters can now be used as a parts set to contribute to the building of future circuits requiring a range of transcription activities.

A.



B.

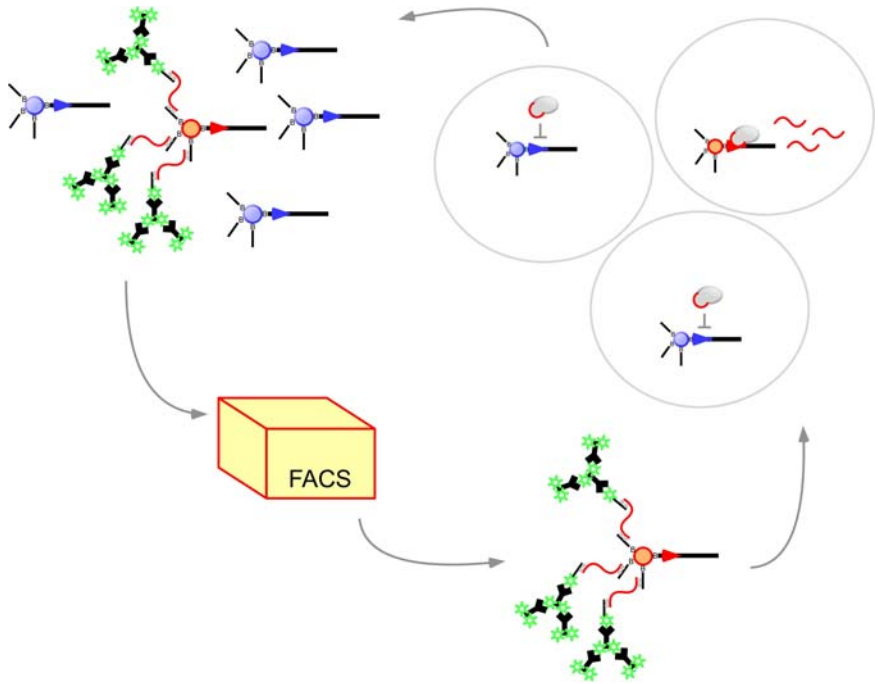


Figure 1. Selection scheme for transcription templates containing active promoter sequences. A. Biotinylated template DNA and biotinylated capture oligonucleotides are immobilized on a streptavidin coated bead. Each bead should one or zero templates and an excess of capture oligonucleotide. Beads are suspended in a transcription reaction and emulsified to encapsulate a single bead per emulsion compartment. RNA produced from the template DNA hybridizes to the bead through the capture oligonucleotide. After breaking the emulsion, the RNA is labeled by a fluorescent probe oligonucleotide and then an antibody based fluorescence amplification system. B. Individual beads are sorted on the basis of their fluorescent signal on an Aria FACS machine. Highly fluorescent beads are collected and reamplified for additional FACS or characterization.

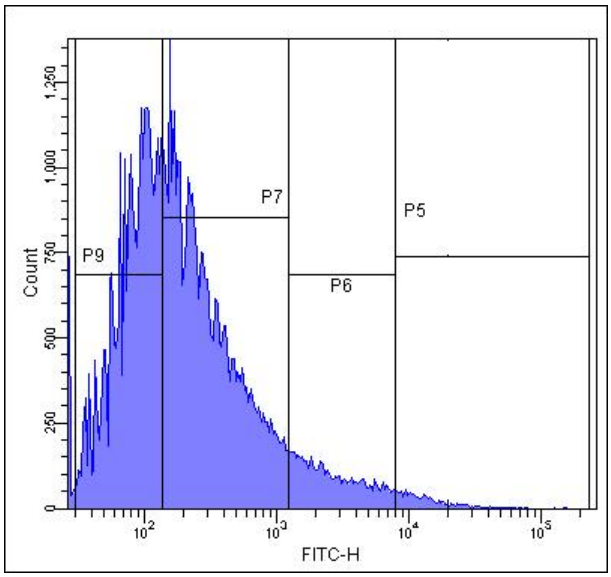
2. Results

2.1. *System design and testing*

We designed a scheme to select for active T7 promoters through emulsified transcription, fluorescence activated cell sorting (FACS) for active templates and then reamplification of collected templates. This process was mediated by attaching biotinylated template DNA to streptavidin coated beads at a ratio of no more than a single template per bead (in practice, no more than a single template for every two beads was added when sorting to minimize beads containing multiple templates). Transcripts produced in emulsion hybridize to the bead containing the template they were transcribed from through a capture oligonucleotide. The RNA remained hybridized to the bead through the process of breaking the emulsion and washing the beads (data not shown). The transcripts are finally fluorescently labeled and beads containing high amounts of fluorescence (and thus active templates) are collected by FACS. The process of preparing beads for emulsified transcription and subsequent fluorescence labeling is presented in Figure 1 and described in detail in the Methods section.

In order to test the viability of this scheme, we investigated its ability to enrich a highly active T7 promoter-containing template from a background of weakly active T7 promoter-containing templates (the weakly active sequence contains +1 C and +2 C instead of +1 G and +2 G in the highly active sequence). The highly active templates were added to a solution of weakly active templates at 1 part in 10 and added to beads at 1 template per 2 beads (thus, at least 50% of the beads should contain no template). During FACS sorting, four non-overlapping regions of the bead population representing nearly the entire fluorescence spectrum were sampled. In order of increasing fluorescence, region P9 contained ~40% of the population, P7 contained ~50% of the population, P6 contained ~7.6% of the population and P5 contained the most fluorescent ~1.6% of the population. The collected

A.

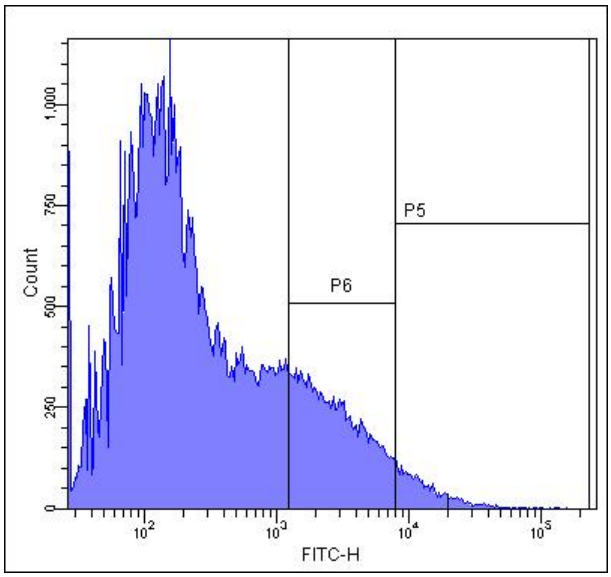


B.

	% of population	active template	weak template
P9	39.5	0	5
P7	50.1	0	5
P6	7.6	4	1
P5	1.6	5	0

Figure 2. One round test selection with a two member population. A. Histogram showing bead distribution by relative fluorescence intensity. The beads carry a two member mixed population of either highly active or weakly active transcription templates. The highly active templates were present at 10% of the total mixed population. Templates were added to the beads at a ratio of 1 template per 2 beads. The total bead population was gated into 4 non-overlapping subpopulations named P9, P7, P6 and P5. B. Population analysis and sequence results show that an increase in relative fluorescence corresponds to an increase in the fraction of highly active template sequences.

A.



B.

Round 3 (P5)	Sequence	Relative transcription efficiency
R3P5 #9	TAATACGACTCACTATA **GGCGG~** TTCCCCATCTTA	100%
R3P5 #8	TAATACGACTCACTATA **GGTAGC** TTCCCCATCTTA	29%
R3P5 #1	TAATACGACTCACTATA **GAGAAT** TTCCCCATCTTA	71%
R3P5#10	TAATACGACTCACTATA **GACTCC** TTCCCCATCTTA	36%
R3P5 #5	TAATACGACTCACTATA **GCTCA~** TTCCCCATCTTA	30%
R3P5 #6	TAATACGACTCACTATA **GTGGAA** TTCCCCATCTTA	89%
R3P5 #2	TAATACGACTCACTATA **AGGGGT** TTCCCCATCTTA	62%
R3P5 #7	TAATACGACTCACTATA **AGATAT** TTCCCCATCTTA	56%
Round 3 (P6)		
R3P6 #5	TAATACGACTCACTATA **TTCAAA** TTCCCCATCTTA	0.3%
R3P6 #2	TAATACGACTCACTATA **CTTCCC** TTCCCCATCTTA	0.5%

Figure 3. A. Histogram showing bead distribution by relative fluorescence intensity for the lib1 Round 2 population. Beads from P5 and P6 gates were collected and resulting sequences analyzed. B. Sequence and relative transcription data from select clones recovered from Round 3 of FACS. Deletions from the expected sequence are represented by a tilde (~). The region randomized in the starting sequence library is shown in bold (generally +1 to +6). Transcription efficiency is shown relative to the most transcribed clone. Sequences were selected for further characterization by *in vitro* transcription assay in order to test a variety of +1 and +2 sequence combinations. All sequences from P5 contained +1 purine +2 N (where N is any of the 4 standard nucleotides). Two clones from P6 that did not contain the +1 purine and were predicted to be inactive were tested and found to have only trace activity.

beads from these regions were amplified, cloned and 5 colonies from each region sequenced to determine the template distribution within the spectrum (Figure 2). Consistent with the addition of only 1 template for every two beads, region P9 amplified poorly compared to the other three regions. All 5 clones from region P5 and 4 of the 5 clones from region P6 were the highly active template sequence. All clones from regions P7 and P9 (together, the least fluorescent ~90% of the bead population) were weakly active templates. These results show that highly active promoter sequences can be differentially identified from a background of weakly active templates.

2.2. *Promoter selection from randomized ITS library*

After confirming that the selection scheme was viable, we wanted to select for active promoter sequences from a randomized promoter library. Starting with the core T7 phi10 promoter sequence (from -17 to -1) we completely randomized the first six bases of the initially transcribed region (ITS positions +1 to +6; lib1). These bases are known to influence promoter strength by affecting the ability of the T7 RNA polymerase to transition from the promoter recognizing initiation complex to the highly processive elongation complex[14]. This is a small library that

can be easily screened by FACS (a theoretical library size of 4^6 or 4096 possible unique members) but that we predicted would retain a broad range of activity due to the inclusion of the -17 to -1 core promoter sequence.

Similar to the initial two member test pool, the lib1 pool was attached to beads at a ratio of 1 template per 2 beads with excess capture oligonucleotide. The library was emulsified for transcription and the resulting RNA labeled beads were fluorescently labeled. Two rounds of FACS enrichment for the highest ~1.5% (round 1) and ~5% (round 2) of the population yielded the Round 2 population seen in Figure 3A. A third round of FACS was performed and two non-overlapping regions were collected (P6 and P5; Figure 3A). The templates from these regions were cloned and 9 sequences for P5 and 10 sequences for P6 were determined. Figure 3B contains a select list of sequences and relative transcription activities. For a full list of sequences, see Appendix Table A1.

The templates which were tested by *in vitro* transcription from Round 3 region P5 (R3P5) contained a broad spectrum of activities down to 30% of the maximum transcription level. The tested promoter sequences were chosen out of the 20 clones sequenced in order to cover clones containing the entire spectrum of nucleotides found at the +1 and +2 positions of the library under the theory that the +1 sequence was the most important for initiation followed closely by the +2 sequence. This expectation appears to have been borne out by the presence of a purine at +1 in 16 out of the 20 total sequences from R3P5 and R3P6 combined. Two of the four sequences that did not contain a purine at +1 were also tested by *in vitro* transcription and found to be inactive, suggesting that further enrichment of the selected pool would likely have been possible. Interestingly, the +1 and +2 positions do not appear to solely control promoter strength; variants in which these residues were the same but where the +3 to +6 sequences were different had quite different transcription activities.

3. Discussion

A number of synthetic genetic circuits have previously been designed and assayed in the context of organisms. In many instances, the overall function of these circuits had to be optimized by varying the levels of gene expression of one or more specific components. In such circuits, subtle variation of individual parts can lead to global changes in circuit behavior. Anderson et al. approached this problem by randomizing the ribosome binding site and selecting for the desired system behavior[1]. Another approach has been to screen through a small number of parts of varying strength to find one suitable for a specific application. Alper et al. used this strategy to identify promoters of varying strength to optimize metabolic pathway output. While library members spanned ~200 fold activity, changes in promoter strength of less than 10 fold were sufficient to optimize metabolite output[15]. Since the performance of biological parts in organisms is idiosyncratic, these optimizations have so far been largely empirical. To date, it has been difficult to match the actual and predicted performances of biological parts in circuits.

This problem is further confounded for synthetic biologists because a few, highly characterized parts, such as the lac and tet promoters, have typically been used again and again. More recent synthetic biology[16] and metabolic engineering[15] efforts have attempted to fine tune promoter strengths, either by screening or by design. Even greater standardization has been achieved by Kelly et al.,[17] who compared the activities of a series of *E. coli* promoters in the context of a single expression casette (GFP) *in vivo*. The establishment of such a standardized system ultimately allows the direct comparison of promoters from different labs or different constructs.

Unfortunately, such efforts will not translate to *in vitro* genetic circuits, and therefore we attempted to recapitulate such standardization in the context of *in vitro* compartments. We chose to standardize transcription using the highly active T7 RNAP and its widely used promoter. Given that the activities of proteins and regulatory sequences in emulsions can be very different from *in vivo* or even *in vitro*, we could not just assume that the previous rule sets for T7 RNA polymerase promoters would be operative in emulsion microvessicles. Indeed, we have previously found that T7 RNA polymerase loses activity in emulsions relative to *in vitro* (data not shown).

We therefore directly selected for different levels of function *in vitro* by modulating the short sequence of the initially transcribed (ITS) region and thereby the promoter strength. Promoter variants were emulsified and differing transcription activities were directly selected by FACS. We initially focused on the most highly active fraction of promoters, although a wide range of transcription activities is obviously possible. The relative transcription activities of ten different selected promoters were determined both in emulsions (via FACS) and in

transcription reactions *in vitro*. The strongest promoters generally had a guanine at +1 and favored G or A at +2, which was expected based on the known strengths of T7 TNAP promoter variants. However, we unexpectedly selected two promoters that initiated with AG, as opposed to all known phi10 promoters that initiate with G. This necessarily expands the rules for T7 RNAP promoter design *in vitro*. The 8 most active promoters spanned a dynamic range of approximately 3-fold.

The definition of a wide range of promoters with varying sequences and activities will provide a unique tool set for the construction of synthetic genetic circuits *in vitro*. The fact that only 6 residues need be varied to vary transcription is reminiscent of the varying translation by simply modulating short ribosome-binding sites, and the two techniques can obviously be used together to exquisitely control the strength of gene expression. While we have begun to standardize, characterize, and compare these new promoter parts, their behaviors can be even more accurately represented once they are tested by predictive models in complex systems.

4. Materials and Methods

4.1. *Template and library construction*

Oligonucleotides were ordered from IDT or, in the case of oligonucleotides with randomized positions, produced in house. For a full list of oligonucleotide and template sequences, see Appendix Table A2. PCR amplifications were performed with Taq DNA polymerase. For bead based experiments and sorting, ED.5Bio template F and ED.Pt7.temp R primers were used. For non-emulsion transcriptions and for cloning, ED.template F was substituted. To construct the ITS library (lib1), the template was amplified with ED.lib1 F and ED.Pt7.temp R. The product of this PCR was agarose gel purified and amplified with ED.lib1build F and ED.Pt7.temp R. The product of this PCR was gel purified and amplified with ED.5Bio template F and ED.Pt7.temp R for immobilization on streptavidin beads.

4.2. *Compartmentalized reactions*

The protocol used for this work was adapted from reference 10. All steps prior to emulsification were performed on ice and all bead centrifugation steps were carried out at 4C and 6,000 RPM for 5 minutes unless otherwise specified. See Appendix A3 for solution recipes.

20 microliters of 1 micrometer diameter streptavidin coated beads (or approximately 4E8 individual beads; Bang Laboratories) was added to 180 microliters PBSTE. The solution was gently mixed and the beads pelleted by centrifugation. The beads were resuspended in 50 microliters of PBSTE with approximately 1 template molecule for every 2 beads and incubated at room temperature for 15 minutes. 3 microliters of 20 micromolar ED.5bio.handle was added and incubated for an additional 45 minutes. The beads were centrifuged and the pellet washed with 100 microliters PBSTE once. This step was repeated two additional times but the beads were washed with 100 microliters of transcription wash buffer.

The oil phase was assembled by adding 500 microliters of oil mixture to a 13mL (95 x 16.8 mm) Starstedt polypropylene tube containing a Spinplus (9.5 x 9.5 mm Teflon) stir bar and placed in a beaker of ice on a magnetic stir plate. The bead pellet was resuspended in a 200 microliter transcription reaction immediately prior to emulsification. The transcription reaction was added in ~10 microliter aliquots over 3 minutes to the stirring oil mixture. The total stir time was 5 minutes. The reaction was transferred to a 1.5 milliliter Eppendorf tube and incubated at 37 degrees.

The transcription reaction was terminated by incubating the reaction on ice for 10 minutes with the addition of 40 microliters of 500 millimolar EDTA and 160 microliters of PBSTEBB. The emulsion was broken by the addition of 0.7 milliliters of diethyl-ether followed by thorough mixture and centrifugation at 13,000 RPM for 4 minutes at 4C. Occasionally the beads would fail to precipitate at this step and remain in the aqueous solution. In this event, the ether phase was removed, 300 microliters of PBSTE was added, gently mixed and centrifuged again. The failure of beads to centrifuge in the first step was not seen to affect the quality of the fluorescent signal.

Once the beads were pelleted, the aqueous phase was removed. The beads were washed in 100 microliters PBSTEBB 3 times before a final resuspension in 200 microliters of PBSTE.

4.3. *Bead labeling and FACS analysis*

3 microliters of 100 micromolar ED.5Fam3.probe was added and incubated on ice for 30 minutes. This fluorescently labeled DNA oligonucleotide hybridizes to the 5' half of the RNA transcript (the 3' half of the RNA hybridizes to the biotinylated capture oligonucleotide). The beads were washed twice in 100 microliters PBSTEBB to remove non-hybridized ED.5Fam3.probe. The fluorescence from the first labeling was insufficient to differentiate active from inactive beads by FACS so an antibody based fluorescence amplification reagent was utilized (Alexa Fluor 488 Signal Amplification Kit, Invitrogen). The beads were resuspended in 200 microliters Antibody Labeling Buffer with 10 milligrams of rabbit anti-fluorescein antibody and incubated for 20 minutes on ice, followed by two 100 microliter PBSTEBB washes. The beads were then resuspended in 200 microliters Antibody Labeling Buffer with 10 milligrams of goat anti-rabbit IgG antibody and incubated on ice for 20 minutes. The beads were washed twice in 200 microliters of PBSTEBB and finally resuspended in 100 microliters PBS.

4.4. *Compartmentalized selection*

Fluorescence based bead sorting was performed on a BD FACSAria Cell Sorting System. Beads were diluted additionally in PBS to an appropriate concentration for sorting. Fractions were collected and amplified by PCR for additional sorting and for cloning and sequencing, as seen in Figure 2 and Figure 3.

4.5. *Cloning and sequencing*

After amplification of templates from sorted beads, PCR products were cloned using a Topo TA cloning kit (PCR4topo, Invitrogen). Individual colonies were chosen for sequencing and to generate PCR templates for transcription analysis.

4.6. *Transcription analysis*

In vitro transcription was performed on selected clones. Reactions were setup similar to the selection protocol, except 0.01 mCi of alphaP^{32}-ATP was added, the template was not immobilized and the total reaction volume was 20 microliters. 50 nanograms of a PCR template was added to each transcription and incubated at 37 degrees for 40 minutes. The reaction was stopped by adding 20 microliters of 18 millimolar EDTA in formamide and heated at 90 degrees for 10 minutes. Transcription products were run on a denaturing (7 M urea) 8% polyacrylamide gel which was then dried and analyzed using a Phosphorimager (Molecular Dynamics).

Acknowledgments

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Appendix

Table A1. Sequences cloned from R3P6 and R3P5 of the Lib1 selection.

R3P5	
R3P5- 9	TAATACGACTCACTATAGGCGG~TTCCCC
R3P5- 8	TAATACGACTCACTATAGGTAGCTTCCCC
R3P5- 1	TAATACGACTCACTATAGAGAATTTCCCC
R3P5- 10	TAATACGACTCACTATAGACTCCTTCCCC
R3P5- 5	TAATACGACTCACTATAGCTCA~TTCCCC
R3P5- 6	TAATACGACTCACTATAGTGGAATTCCCC
R3P5- 4	TAATACGACTCACTATAGTAGTATTCCCC
R3P5- 2	TAATACGACTCACTATAAGGGGTTTCCCC
R3P5- 7	TAATACGACTCACTATAAGATATTTCCCC
R3P6	
R3P6- 7	TAATACGACTCACTATAGGATTGTTCCCC
R3P6- 3	TAATACGACTCACTATAGAATAATTCCCC
R3P6- 1	TAATACGACTCACTATAGCTCACTTCCCC
R3P6- 8	TAATACGACTCACTATAGCAACTTTCCCC
R3P6- 9	TAATACGACTCACTATAGCACCCTTCCCC
R3P6- 4	TAATACGACTCACTATAAGTCGCTTCCCC
R3P6- 2	TAATACGACTCACTATACTTCCCTTCCCC
R3P6- 6	TAATACGACTCACTGTACTTAGATTCCCC
R3P6- 10	TAATACGACTCACTATACTTAGATTCCCC
R3P6- 5	TAATACGACTCACTATATTCAAATTCCCC

Table A2. Template and oligonucleotide sequences.

Name	Sequence
highly active template	GTCGACAAGCTTGCGGCCGCATAATGCTTAAGTCGAACAGAAAGTAATCGTATTGTACACGGCCGCATAATCGAAAT taatacgactcactatagggaatTCCCCATCTTAGTATATTAGTTAAGTATAAGAAGGAGATATACATATGAGCCA TATTCAACGGGAAACGTCTTGCTCTAGGCCGCGATTAAATTCCAACATGGATGCT
weakly active template	GTCGACAAGCTTGCGGCCGCATAATGCTTAAGTCGAACAGAAAGTAATCGTATTGTACACGGCCGCATAATCGAAAT taatacgactcactatacctattTTCCCCATCTTAGTATATTAGTTAAGTATAAGAAGGAGATATACATATGAGCCA TATTCAACGGGAAACGTCTTGCTCTAGGCCGCGATTAAATTCCAACATGGATGCT
lib1 template	GTCGACAAGCTTGCGGCCGCATAATGCTTAAGTCGAACAGAAAGTAATCGTATTGTACACGGCCGCATAATCGAAAT taatacgactcactatannnnnnTTCCCCATCTTAGTATATTAGTTAAGTATAAGAAGGAGATATACATATGAGCCA TATTCAACGGGAAACGTCTTGCTCTAGGCCGCGATTAAATTCCAACATGGATGCT
ED.lib1build F	GTCGACAAGCTTGCGGCCGCATAATGCTTAAGTCGAACAGAAAGTAATCGTATTGTACACGGCCGCATAATCGAAATtaatacgactcac
ED.5bio template F	Bio-GTCGACAAGCTTGCGGCCGCATAATG
ED.template F	GTCGACAAGCTTGCGGCCGCATAATG
ED.PT7.temp R	AGCATCCATGTTGGAATTTAATCGCGGCCTAGAGC
ED.5Fam3.probe	6Fam-AGAGGTTTCCGTTGAATATGGCTCATATGTATATC-6Fam
ED.5bio.handle	Bio-AGCATCCATGTTGGAATTTAATCGCGGCCTAGAGC
ED.lib1 F (ITS)	ATCGAAATtaatacgactcactatannnnnnTTCCCATCTTAGTATATTA

A3. Solutions.

PBSTE	10mM	NaPO4 pH 7.4
	150mM	NaCl
	0.1%	Tween-20
	10mM	EDTA
PBSTEB	PBSTE plus 0.1% BSA	
PBSTEBB	PBSTE plus 0.1% BSA and 100uM biocytin	
Antibody Labeling Buffer	PBSTE plus 100uM biocytin, 1% BSA, 100ug tRNA (1uL), 80U RNAsin (2uL) and 10ug Antibody (5uL) per 200uL	
Transcription Buffer	50mM	EPPS pH 8.0
	2mM	Spermidine
	10mM	DTT
	50mM	KCl
	30mM	MgCl2
Transcription Mix	200uL total Transcription buffer plus 4U yeast pyrophosphatase, 160U RNAsin Plus, 1000U T7 RNAP and 2mM NTPs	
Transcription Wash Buffer	Transcription buffer plus 0.1% BSA	
Oil Mixture	475uL	Mineral Oil
	22.5uL	Span 80
	2uL	Tween 80
	0.25uL	Triton X-100

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