**File Name:** Supplementary Information  
**Description:** Supplementary Figures, Supplementary Tables and Supplementary References

**File Name:** Peer Review File  
**Description:**
Supplementary Figure 1. Segment-by-segment saturation mutagenesis of \( P_{ECF11} \) on a medium-copy-number backbone.

Each dot represents a randomly selected mutant containing mutations in the corresponding sequence segment. The promoter activity was measured using superfolder GFP as the reporter and quantified as the arithmetic mean of flow cytometry fluorescence data. CV, coefficient of variation; FV, relative (-fold) variation determined by the ratio of maximal promoter activity to the minimal observed value. Data represent the averages of at least three replicate experiments conducted on different days.
Supplementary Figure 2. Genetic Refinement of Additional ECF and T7-Family Promoters. (a) Effect of random mutagenesis on the promoter activity of P_{T3}. (b) Effect of random mutagenesis on the promoter activity of P_{MmP1}. (c) Effect of random mutagenesis on the promoter activity of P_{gh-1}. (d) Effect of random mutagenesis on the promoter activity of P_{ECF16}. (e) Effect of random mutagenesis on the promoter activity of P_{ECF20}. Each dot represents a randomly selected mutant containing mutations in the corresponding sequence segment. The promoter activity was measured using superfolder GFP as the reporter and quantified as the arithmetic mean of flow cytometry fluorescence data. CV, coefficient of variation; FV, relative (-fold) variation determined by the ratio of maximal promoter activity to the minimal observed value. Data represent the averages of at least three replicate experiments conducted on different days.
Supplementary Figure 3. Effects of activator expressions on the growth rate of *E.coli*. (a) Effect of T7 RNAP and its sfGFP reporter. (b) Effect of σ^{ECF11} and sfGFP reporter. The relative growth rate was obtained by extracting the “cell event number per ten seconds” from the flow cytometry data of each measurement. Data represent the means ± SD from at least three replicate experiments conducted on different days.
Supplementary Figure 4. Experimental Measurements and Parameter Fitting of Response Functions for cI434 acting on P_{T3} (a) and P_{gh-1} (b). Solid lines represent the data of parameter fitting using a non-equilibrium correction term. Open circuits represent experimental measurements. $\delta_R$ values for each response function obtained from model fitting are given. The promoter activity was calculated as the arithmetic mean of flow cytometry fluorescence data using sfGFP as the reporter. Data represent the means ± SD from at least three replicate experiments conducted on different days.
Supplementary Figure 5. Raw Data and Model Fitting for T7-RNAP Promoter Cores and 32 Repressor-Operator Pairs. (a) Transcriptional activity of T7-RNAP and ECF11 promoter cores. Promoter cores described in Fig. 4 are highlighted in red and those used for the fitting of repression-dependent parameters are highlighted in blue. The corresponding sequences of the promoter cores are listed in Supplementary Table 3. (b) Experimental measurements and parameter fitting of response functions for 32 repressor-operator pairs. The response functions for the four operators targeted by cI434 are shown in Fig. 3. Open circles and solid lines represent experimental data and model fitting results, respectively. Data represent the means ± SD from at least three replicate experiments conducted on different days.
Supplementary Figure 6. Experimentally measured and predicted response functions of 107 Combinational Promoters Selected for Genetic Implementation. Boxed in red are the combinational promoter designs using cymR as the repressor which were selected as a group. Others were selected randomly. Mean relative (-fold) errors for each response function and the corresponding promoter design are given. The promoter activity was measured using superfolder GFP as the reporter and quantified as the arithmetic mean of flow cytometry fluorescence data. Data represent the means ± SD from at least three replicate experiments conducted on different days.
**Supplementary Figure 7. Parameterization of P_{TAC} Promoter Variants.** (a) Response functions of P_{TAC} promoter variants using IPTG concentrations as the input. The solid curves represent the fitting results according to the biophysical model in (c). Data were obtained by measuring the flow cytometric fluorescence of cells harboring pRG-sfGFP under the control of wild-type and mutant P_{TAC} promoters. Error bars represent the standard deviations from at least three biological replicates. (b) Sequence alignment of P_{TAC} promoter variants. Mutations are marked in red. (c) The biophysical model used to fit the response functions of P_{TAC} promoter variants. $K_I$ and $n_I$ represent the dissociation constants for IPTG and LacI, respectively; $\alpha$ and $\beta$ denote the maximal and basal promoter activity, respectively; $K_{lac}$ is the constant for LacI binding to lacO. (d) Parameter database of P_{TAC} promoters. The output of a P_{TAC} promoter carried on p15A-AmpR (pRG plasmid) is the input of transcriptional repression and the output of a P_{TAC} promoter on the chromosome is the input of transcriptional activation.
Supplementary Figure 8. Detailed Fitness Landscape of all 30,528 Network Designs. $P_{TAC}$ for repressors: the promoter controlling the expression of repressors; $P_{TAC}$ for activator: the promoter controlling the expression of T7 RNAP.
Supplementary Figure 9. Experimentally determined and predicted response functions of IFFL networks using cI434 as the repressor. (a) IFFL network based on the operator O_{4-cI434}. (b) IFFL network based on the operator O_{3-cI434}. NE+, with non-equilibrium correction term; NE-, without non-equilibrium term. MFE, mean relative (-fold) error. A secondary increase of GFP expression attributed to the non-equilibrium term is indicated. The output, GFP fluorescence, was measured using flow cytometry and quantified as the arithmetic means of measurements. Data represent the means ± SD from triplicate experiments conducted on different days.
Supplementary Figure 10. Experimental and predicted response functions of re-designed IFFL networks. The wild-type T7 promoter of the highest-fitness IFFL network was replaced by five new promoter cores. (a) Schematic representation of network link assignment, design space and computation task. (b) Experimental and predicted response functions of IFFL networks using PT7M1, PT7M3, PT7M13, PT7M14 and PT7M16 as the promoter cores instead of PT7wt. The dashed line indicates the peak position of the highest-fitness IFFL circuit in Fig. 6f. Data represent the means ± SD from three replicate experiments conducted on different days.
Supplementary Figure 11. Emergent Transcriptional Activity in Two Failed Combinatorial Promoter Designs. (a) Spontaneous (emergent) transcriptional activity of promoter cores, operators and their combinations in the absence of T7 RNAP. (b) Experimental and predicted response functions for two failed promoter designs before and after integrating the contribution of emergent promoter activity. MEF was calculated as described in Fig. 4. The promoter activity was calculated as the arithmetic mean of flow cytometry fluorescence data using sfGFP as the reporter. (c) Spontaneous Transcriptional Activity of all 83 Combinational Promoters in the Absence of T7 RNAP. The two combinational promoters shown in (b) are highlighted in green. The promoter activity was measured using superfolder GFP as the reporter and quantified as the arithmetic mean of flow cytometry fluorescence data. Data represent the means ± SD from at least three replicate experiments conducted on different days.
Supplementary Figure 12. Plasmid Architecture of pPT and pRG. The vast majority of the plasmids used in this study were derived from two basic vectors: pPT and pRG. We named the derived plasmids according to the schemes “pPT-XYZ” and “pRG-XYZ”, whereby XYZ denotes the sequence of interest by which the lacZα fragment was replaced via Golden Gate Assembly. The crucial parts of the plasmid backbones are labeled with Roman numerals and identical parts are labeled with the same number. The corresponding sequences are summarized in Supplementary Table 4.
Supplementary Table 1. Sequences of Promoters, Single Operators, RNAPs, ECF σs and Transcriptional Repressors.

| Name | Promoter/Operator | RNAP/ECF σ/ Transcriptional Repressor |
|------|-------------------|---------------------------------------|
| T7   | TAATACGACTCTACATA | T7 TAATACGACTCTACATA GGGG             |

*σ*: ECF11
| MmP-1 | GTTGGCAGACGCTGTAATGGCAGCACACTTTTGGGCAATGTTGGGTAATGGCAATCCTGCAGGACCAGCTGCACGAATCTCAGCTGGACAAAATGCCGCCGATCCCGGAAATGGGTACCTGGGACATTCGCTTCCGGAAGATCAGCGGAGCTTTGTCATTCCTGAGTGGCTGGACATCCGTGAAATCCTGAAATCTCAGTTCGCTTTCGCTTAA |
| T3  | ATTAACCCCTACTAAAGGGAACATTTTTTGGGCAATGTTGGGTAATGGCAATCCTGCAGGACCAGCTGCACGAATCTCAGCTGGACAAAATGCCGCCGATCCCGGAAATGGGTACCTGGGACATTCGCTTCCGGAAGATCAGCGGAGCTTTGTCATTCCTGAGTGGCTGGACATCCGTGAAATCCTGAAATCTCAGTTCGCTTTCGCTTAA |

**GTTCGATC**
ATGCTTGGACGTCAAGCCGAAAGCTGAGATTGCAGACAACGCAGCCGCTAAGCGTACTCCGCTACGCTTCTCCCTAAGTTAACCACACGTATCGTCGAGTGGCTCGAAGAGTACGCATCGAAGAAAGGCCGCAAGCCTAGCGCATACGCACCGCTCCAGTTACTCAAGCCGGAGGCCTCCGCGTTTATCACCCTGAAAGTTATCCTTGCGTCACTAACCAGTACGAACATGACAACCATTCAGGCCGCTGCTGGTATGCTGGGGAAAGCCATTGAGGACGAGGCACGATTTGGGCGCATCCGTGACCTAGAAGCGAAGCACTTCAAGAAGCACGTTGAGGAACAGCTTAACAAGCGCCACGGGCAAGTCTACAAGAAAGCATTTATGCAGGTGGTCGAGGCCGATATGATTGGTCGAGGTCTGCTTGGTGGCGAGGCGTGGTCTAGCTGGGATAAAGAAACCACGATGCACGTAGGGATTCGCCTGATTGAAATGCTGATTGAATCCACGGGTCTGGTGGAATTACAGCGCCACAACGCAGGTAACGCAGGCTCTGACCATGAGGCACTGCAACTGGCCCAAGAGTACGTGGACGTATTAGCGAAGCGTGCAGGCGCTCTGGCGGGTATCTCTCCGATGTTCCAGCCGTGTGTCGTACCGCCGAAACCTTGGGTAGCAATCACAGGGGGCGGCTATTGGGCTAACGGTCGCAGACCTTTGGCACTCGTTCGCACTCACTCTAAGAAGGGCTTGTAGCGCTACGAAGACGTTTACATGCCAGAAGTCTACAAGGCTGTGAACCTCGCGCAAAACCCGCATGGAAAATCAACAAGAAAGTTCTTGCTGTTGTCAATGAGATTGTTAACTGGAAGAATTGCCCGTAGCAGACATTCCATCGCTGGAGCGCCAAGAGTTACCGCCTAAGCCTGACGACATTGACACCAACGAGGCAGCGCTCAAGGAGTGGAAGAAAGCCGCTGCTGGTATCTATCGCTTGGACAAGGCACGAGTGTCTCGCCGTATCAGCTTAGAGTTCATGCTGGAGCAGGCCAACAAGTTCGCAAGTAAAGAAAGCAATCTGGTTCCCTTACAACATGGACTGGCGCGGTGTCGTGTACGCTGTGCCGATGTTCACCCGCAAGGCAACGACATGACGAAAGGTCTGCTGACCCTTGCTAAAGGCAAGCCAATCCTTGAACCTTGGAGGCTGAGCAGGATTCACCGTTCTGTTTCCTCGCGTTTTGCATTGCAGGCGTTACGCACCACGGTCTGAGCTACAATTGCTCTCTGCCGCTGGCTTCCAGGAAGTTGAATCTGCTGAAGCTTCTCGTGACCTGACCAAACTGCTGAAACAGCTGCCGGACGTCAGCGTCTGCCGATCGTTCACGTTAAACTGGAAGGTCTGTCTGTTGAAGAAACCGCTCA

σECF16

GTAACCCTGGCGGCAGSAACAGCAAGAATCTCAACTGCAAAGACCACTTCAAGTCTCCGCTGCGTGCTGCTG

CAGGACGTGTCCTGACCAAC

17
| Sigma | Consensus Sequence |
|-------|--------------------|
| ECF20 | AGCGATCCTCCGCCCAT |
| CI434 | TACAAGAAGATTTGGT |
| HKCI  | TGAACCATAGTCCA |
| P22C2 | ATTTAAGTGTTCTTTAA |
| TP901C1| AGTTTATGACGTCGAA |
| Protein  | DNA Sequence  |
|----------|---------------|
| Cro      | GGTATCAAGCTGAGTAAAAGTACGCTGTCGCAGTATGTGAACTCTGTGCAGTCGCCAGACCAGAACCGCATCTATTTACTGGCGAAAACGCTGGGCGTTTCCGAAGCTTGGCTGATGGGATTTGATGTTCCAATGGTTGAATCGTCGAAAATCGAAAACGATTCGGAAAACATCGAAGAAACCATTACG |
| Mnt      | AGGTCCACGGTGGACCTTACTAGAAAGTCTATAATACTAGATGGCCCGGGATGATCCTCACTTCAATTTTCGTATGCCATGGAAGTAAGAGAGAAATTGAAATTTAGAGCAGAGGCAAACGGACGGAGCATGAACTCTGAGCTTTTGCAAATCGTACAAGATGCCCTAAGCAAACCGTCACCAGTCACTGGGTACCGCAATGATGCGGAACGACTCGCCGATGAGCAGAGCGAGTTAGTGAAGAAGATGGTCTTCGATACACTGAAGGATC TTATATAAAAAACCACCTGA |
| TetR     | TCCCTATCAGTGATAGATATCGACAGTCATGTTAAAACACGCTGCTAAAGAAGAAAGGGAAACCACTACTGATAGTATTGCCGCCATTATTACGACAAGCTATCGAATTATTTGATCACCAAGGGCAGAGCCAGCCTTCTTATTCGGCCTTGAATTGATCATATGCGGATTAGAAAAACAACTTAAAAGTGAAAGTGGGTCCTGAGAAGTGCGAGTCAGGTAGCTGA |
| mTetR    | TCCCCGTCAGTGACGGAAGTCTCTCTAAATACTAGATGGTAATAATGAGCCCAAAAAGAAGAACACAAGCAGAAAGAGCAATGGAAACACAAGGAAAACTAATAGCAGCAGCACTAGGAGTACTAAGAGAAAAAGGAACGCAGGATTCAGAATAGCAGACGTACCAGGAGCAGCAGGAGTAAGCAGAGGAGCACAAAGCAGCACCACTTCCCAACAAAACTAGAACTACTACTAGCAACATTCGAATGGCTATACGAACAAATAAACAGAAAGCAGAGCAAGACTAGCAAAACTAAAACCAGAGGACGACGTAATACAACAAATGCTAGACGACGCAGCAGAGTTCTTCCTAGACGACGACTTCAGCATAAGCCTAGACCTAATAGTAAGCAGCAGACCGCGACCCAGCACTAAGAGAAGGAATACAAAGAACAGTAGAAAGAAACAGATTCGTAGTAGAGGACATGTGGCTAGGAGTGCTAGTAAGCAGAGGACTAAGCAGAGACGACGCAGAGGACATACTATGGCTAATATTCAACAGCGTAAGAGGACTAGCAGTAAGAAGCCTATGGCAAAAA |
| CymR     | ACAAACAGACAACATCTGGAAGTGGTTTGTATTAGTGGTAAAAGTACGCTGTCGCAGTATGTGAACTCTGTGCAGTCGCCAGACCAGAACCGCATCTATTTACTGGCGAAAACGCTGGGCGTTTCCGAAGCTTGGCTGATGGGATTTGATGTTCCAATGGTTGAATCGTCGAAAATCGAAAACGATTCGGAAAACATCGAAGAAACCATTACG |
| Aga      | ACAAAGAAGCTGAAAGTACGCTGTCGCAGTATGTGAACTCTGTGCAGTCGCCAGACCAGAACCGCATCTATTTACTGGCGAAAACGCTGGGCGTTTCCGAAGCTTGGCTGATGGGATTTGATGTTCCAATGGTTGAATCGTCGAAAATCGAAAACGATTCGGAAAACATCGAAGAAACCATTACG |
| **PhlF** | ATGATAGGAAACGTACCCTTGGAAATCTATAGAAGTACCCCGAGCTAGCACATTTCAATTGGCACGTTAGCTGAGGCTTGTATTACGGAAGTGGGAGCCGAGCTCAGGAACCCGTCTGTTTCAGCTGCAGGGTTATTATGGCACCGGTCTGTATCAGATTATCAAAGAAGCGGTAACCCGAAAGGTAGCCTGTATTATCATTTTCCGGGTGGTAAAGAACAGCTGGCAATTGAAGCAGTGAACGAAATGAAAGAATATATCCGCCAGAAAATCGCCGATTGTATGGAAGCATGTAACCGGATCCGGCAGAAGGTATTCAGGCATTTCTGAAAGAACTGAGCTGTCAGTTTAGCTGTACCAGAAGATATTGAAGGTCTGCCGGTTGGTCTGCTGGCAGCAGAAACCAGCCTGAAAAGCGAACCGCTGCGTGAGAAGCATGTCATGAAGCATATAAAGAATGGGCCAGCGTGTATGAAGAAAAACTGCGTACAGACCGGTTGTAGCGAAAGCCGTGCAAAAGAAGCAAGCACCGTTGTTAATGCAATGATTGAAAGGTGGTATTCTGCTGAGCCTGACCGCAAAAAATAGCACACCGCTGCTGCATATTAGCAGCTGTATTCCGGATCTGCTGAAACGTGGTGGCGGTGGCAGCGGTGGCGGTGGCAGCAGATTTGTTGGGACGGTACGTTAGCCCAAAGGGAAATATCCATTGATTAGCATGGTTAGAGCTGGTTCGTGGTGTAAGWCTTGTGAACCCTACGATATCAAGGACATTGATGAATGGTATGACAGTGACGTTAACTTATTAGCAATGGATTCTGGCTGAAGGTTGAAGGTGATTCCATGACCTCACCTGTAGGTCAAAGCATGCCCTGAAGGTCATATGGTGTTAGTAGATACTGGACGGGAGCCAGTGAATGGAAGCCTTGTTGTAAGCACCACACTGACTGACGCGAACGAAGCAACATTCAAGAATACCTGCAATAGATGGCGGTCAGAAGTACCTGAAAGGCCTGAATCCTTCATGGCCTATGACTCCTATCAACGGAAACTGCAAGATTATCGTGGTGTGGAAGCGAGGGTAAAATTCGTATAA |
| **LmrA*** | GATAATAGACACCCTGATATTATT |

a The underlined segments within promoter sequences indicate the promoter cores produced via genetic refinement.
b Protein coding sequences are indicated by capital letters. The CDS of an RNAP may be combined with different RBSs in different scenarios (See Supplementary Tables 5-7).
## Supplementary Table 2 σ^{70}-dependent Promoters Emerging at the Interfaces of Transcriptional Elements.

| Combined Elements | Promoter Prediction^a |
|-------------------|------------------------|
| O_{2/4-AmtR}      | TTTCTATCGCTATAGATAAgctagctacTTTCTATCGCTATAGATAAgctagctacTTTCTATCGCTATAGATAAgctagctac |
| O_{2/4-LmrA^*}    | GATAATAGACCCTGACTATATTtagctagCTAATAGACCGTCACTATATTtagctagCTAATAGACCGTCACTATATTtagctagc |
| P_{T7M43} + O_{2-c434} | atgcctccacaccgctgctcactctctgGACAACGACTCACTATAGGGGTACAAGAAAAAGTTTTGTTgctactCTACAAGAAAAAGTTTTGTTgctactTACAAGAAAAAGTTTTGTTgctact |
| P_{T7M48} + O_{5-TP901c} | atgcctccacaccgctgctcactctctgAGTTGATGAAACGTGAACtctagctagTCATCGACTCACTATAAGGGGTGATGAAACGTGAACtctagCTCATCGACTCACTATAAGGGGTGATGAAACGTGAACtctag |

^a Operators are indicated by plain capital letters; promoter cores are indicated by bold capital letters. The -35 and -10 regions of putative σ^{70}-dependent promoters are underlined.
Supplementary Table 3. Parameter Database of Promoter Cores and Operators.

**Activator: T7 RNAP**

| Name    | Sequence         | $\alpha = 16,462; \beta = 19; n_A = 1.34$ |
|---------|------------------|--------------------------------------------|
| P$_{T7N}$ | TAATACGACTCCTATAGGGG | 0.274 ($K_A = 2,532$)                     |
| P$_{T7M1}$ | TATAACGACTCCTATAGGGG | 0.115                                      |
| P$_{T7M2}$ | GAAAACGACTCCTATAGGGG | 0.104                                      |
| P$_{T7M3}$ | GGATACGACTCCTATAGGGG | 0.0382                                     |
| P$_{T7M4}$ | TAAATACGACTCAGTCAGGGG  | 0.0273                                     |
| P$_{T7M5}$ | GTGCACGACTCCTATAGGGG | 0.00396                                   |
| P$_{T7M6}$ | TAAATACGACTCAGTCAGGGG  | 0.00185                                   |
| P$_{T7M11}$ | ATTTACGACTCCTATAGGGG | 0.230                                      |
| P$_{T7M12}$ | AATACGACTCCTATAGGGG | 0.227                                      |
| P$_{T7M13}$ | GAATACGACTCCTATAGGGG | 0.191                                      |
| P$_{T7M14}$ | TAAACGACTCCTATAGGGG | 0.160                                      |
| P$_{T7M15}$ | TTTAACGACTCCTATAGGGG | 0.144                                      |
| P$_{T7M16}$ | CAAACGACTCCTATAGGGG | 0.142                                      |
| P$_{T7M17}$ | AATTCGACTCCTATAGGGG | 0.137                                      |
| P$_{T7M18}$ | AAAAAACGACTCCTATAGGGG | 0.124                                     |
| P$_{T7M19}$ | AGCTACGACTCCTATAGGGG | 0.117                                      |
| P$_{T7M20}$ | CTAAACGACTCCTATAGGGG | 0.0998                                     |
| P$_{T7M21}$ | ACCTACGACTCCTATAGGGG | 0.0816                                     |
| P$_{T7M22}$ | TAAACGACTCCTATAGGGG | 0.0731                                     |
| P$_{T7M23}$ | ATAAACGACTCCTATAGGGG | 0.0719                                     |
| P$_{T7M24}$ | GTAAACGACTCCTATAGGGG | 0.0706                                     |
| P$_{T7M25}$ | AGATACGACTCCTATAGGGG | 0.0643                                     |
| P$_{T7M26}$ | AATACGACTCCTATAGGGG | 0.0589                                     |
| P$_{T7M27}$ | TGATACGACTCCTATAGGGG | 0.0565                                     |
| P$_{T7M28}$ | AGAAACGACTCCTATAGGGG | 0.0518                                     |
| P$_{T7M29}$ | CTAACGACTCCTATAGGGG | 0.0367                                     |
| P$_{T7M30}$ | CATACGACTCCTATAGGGG | 0.0332                                     |
| P$_{T7M31}$ | ATACGACTCCTATAGGGG | 0.0267                                     |
| P$_{T7M32}$ | ATACGACTCCTATAGGGG | 0.0256                                     |
| P$_{T7M33}$ | GCATACGACTCCTATAGGGG | 0.0247                                    |
| P$_{T7M34}$ | ATCTACGACTCCTATAGGGG | 0.0244                                     |
| P$_{T7M35}$ | ACCTACGACTCCTATAGGGG | 0.0212                                     |
| P$_{T7M36}$ | ACAACGACTCCTATAGGGG | 0.0204                                     |
| P$_{T7M37}$ | TCCTACGACTCCTATAGGGG | 0.0203                                     |
| P$_{T7M38}$ | GCCTACGACTCCTATAGGGG | 0.0199                                     |
| P$_{T7M39}$ | CACTACGACTCCTATAGGGG | 0.0177                                     |
| P$_{T7M40}$ | GCCTACGACTCCTATAGGGG | 0.0169                                     |
| P$_{T7M41}$ | TTGAACGACTCCTATAGGGG | 0.0166                                     |
| P$_{T7M42}$ | CACAAACGACTCCTATAGGGG | 0.0161                                     |
| P$_{T7M43}$ | GACCACGACTCCTATAGGGG | 0.0146                                     |
| P$_{T7M44}$ | TGCCCGACTCCTATAGGGG | 0.0136                                     |
| P$_{T7M45}$ | CTCTCGACTCCTATAGGGG | 0.0113                                     |
PT7M46 | GTGAACGACTCACTATAGGGG | 0.00962
PT7M47 | CGCAACGACTCACTATAGGGG | 0.00956
PT7M48 | CTCACTGACACTATAGGGG | 0.00804
PT7M49 | TTTGTCGACTCACTATAGGGG | 0.00614
PT7M50 | AGCCACGACTCACTATAGGGG | 0.00611
PT7M51 | AGGAACGACTCACTATAGGGG | 0.00575
PT7M52 | TGCAGCGACTCACTATAGGGG | 0.00372
PT7M53 | AATGAGCAGCTCACTATAGGGG | 0.00365
PT7M54 | AAGCCACGACTCACTATAGGGG | 0.00272
PT7M55 | CCGTTCGACTCACTATAGGGG | 0.00237
PT7M56 | CCGACCAGCTCACTATAGGGG | 0.00235
PT7M57 | GTGCAGCGACTCACTATAGGGG | 0.00231

### Operators

| Name     | Sequence<sup>b</sup>                                | $K_R$ | $n_R$ | $\delta_R$ |
|----------|------------------------------------------------------|------|------|------------|
| O<sub>1</sub>-cI434 | [N<sub>21</sub>] TACAAGAAAGTTTGTTCGagttg | 885  | 1.5  | 2811       |
| O<sub>2</sub>-cI434 | [N<sub>21</sub>] TACAAGAAAGTTTGTTCGagttg TACAAGAAAGTTTGTTCGagttg | 379  | 2    | 1141       |
| O<sub>3</sub>-cI434 | TACAAGAAAGTTTGTTCGagttg [N<sub>21</sub>] TACAAGAAAGTTTGTTCGagttg | 410  | 2.7  | 108        |
| O<sub>4</sub>-cI434 | TACAAGAAAGTTTGTTCGagttg TACAAGAAGTTTGTTCGagttg TACAAGAAAGTTTGTTCGagttg | 350  | 3.8  | 64         |
| O<sub>1</sub>-HKcI | [N<sub>21</sub>] TGAACCATAAGTTGCagcttg | 771  | 0.9  | 193        |
| O<sub>2</sub>-HKcI | [N<sub>21</sub>] TGAACCATAAGTTGCagcttg TGAACCATAAGTTGCagcttg TGAACCATAAGTTGCagcttg TGAACCATAAGTTGCagcttg | 42   | 1.8  | 410        |
| O<sub>3</sub>-HKcI | TGAACCATAAGTTGCagcttg [N<sub>21</sub>] TGAACCATAAGTTGCagcttg | 91   | 2.7  | 25         |
| O<sub>4</sub>-HKcI | TGAACCATAAGTTGCagcttg TGAACCATAAGTTGCagcttg TGAACCATAAGTTGCagcttg TGAACCATAAGTTGCagcttg | 50   | 3.2  | 0.1        |
| O<sub>1</sub>-P22c2 | [N<sub>21</sub>] ATTTAAGTGTTCTTTTATCgcttgttcgctgtc | 78   | 1.2  | 400        |
| O<sub>2</sub>-P22c2 | [N<sub>21</sub>] ATTTAAGTGTTCTTTTATCgcttgttcgctgtc ATTTAAGTGTTCTTTTATCgcttgttcgctgtc ATTTAAGTGTTCTTTTATCgcttgttcgctgtc ATTTAAGTGTTCTTTTATCgcttgttcgctgtc | 50   | 4.1  | 70         |
| O<sub>3</sub>-P22c2 | ATTTAAGTGTTCTTTTATCgcttgttcgctgtc [N<sub>21</sub>] ATTTAAGTGTTCTTTTATCgcttgttcgctgtc | 52   | 2.6  | 191        |
| O<sub>4</sub>-P22c2 | ATTTAAGTGTTCTTTTATCgcttgttcgctgtc ATTTAAGTGTTCTTTTATCgcttgttcgctgtc ATTTAAGTGTTCTTTTATCgcttgttcgctgtc ATTTAAGTGTTCTTTTATCgcttgttcgctgtc ATTTAAGTGTTCTTTTATCgcttgttcgctgtc | 42   | 4    | 47         |
| O<sub>1</sub>-TP901cI | [N<sub>21</sub>] AGTTTCATGAAACGTGAACCTgccggtcagtc | 71   | 1.3  | 671        |
| O<sub>2</sub>-TP901cI | [N<sub>21</sub>] AGTTTCATGAAACGTGAACCTgccggtcagtc AGTTTCATGAAACGTGAACCTgccggtcagtc AGTTTCATGAAACGTGAACCTgccggtcagtc AGTTTCATGAAACGTGAACCTgccggtcagtc | 84   | 1.6  | 288        |
| O<sub>3</sub>-TP901cI | AGTTTCATGAAACGTGAACCTagtctagtg [N<sub>21</sub>] AGTTTCATGAAACGTGAACCTagtctagtg AGTTTCATGAAACGTGAACCTagtctagtg AGTTTCATGAAACGTGAACCTagtctagtg AGTTTCATGAAACGTGAACCTagtctagtg | 58   | 1.3  | 379        |
| O<sub>4</sub>-TP901cI | AGTTTCATGAAACGTGAACCTagtctagtg [N<sub>21</sub>] AGTTTCATGAAACGTGAACCTagtctagtg AGTTTCATGAAACGTGAACCTagtctagtg AGTTTCATGAAACGTGAACCTagtctagtg AGTTTCATGAAACGTGAACCTagtctagtg | 78   | 1.5  | 207        |
| Activator: $\sigma^{ECF11}$ | Promoter Cores |
|-----------------------------|----------------|
| $\alpha = 14.206$; $\beta = 7.3$; $n_A = 1.13$ | |

| Activator | Promoter Core | Score |
|-----------|---------------|-------|
| O1-Cro    | [N_{21}]TATCACCAGCAAGGGTAGatcc | 67    | 0.93 | 1879 |
| O2-Cro    | [N_{21}]TATCACCAGCAAGGGTAGatctTATCACCAGCAAGGGTAGatcTATCACCAGGAAGGGTAGatc | 82    | 1.1 | 1754 |
| O3-Cro    | TATCACCAGCAAGGGTAGatctg[N_{21}]TATCACCAGCAAGGGTAGatc | 70    | 0.71 | 1649 |
| O4-Cro    | TATCACCAGCAAGGGTAGatctTATCACCAGCAAGGGTAGatcTATCACCAGCAAGGGTAGatcTATCACCAGCAAGGGTAGatc | 89    | 0.96 | 1600 |
| O1-Mnt    | [N_{21}]AGGTCCACCGGTGACCTtgatc | 128   | 1.6 | 1136 |
| O2-Mnt    | AGGTCCACCGGTGACCTctatg[N_{21}]AGGTCCACCGGTGACCT | 150   | 1.6 | 1218 |
| O1-TetR   | [N_{21}]TCCCTATCAGTGAGATGatctcacacctcttc | 18    | 2    | 81 |
| O4-TetR   | TCCCTATCAGTGAGATGatctcacacctcttcTCCCTATCAGTGAGATGactcacacctcttcTCCCTATCAGTGAGATGatctcacacctcttcTCCCTATCAGTGAGATGactcacacctcttc | 20    | 2.7 | 0.1 |
| O1-mTetR  | [N_{21}]TCCCGcTCAGTGAGACGAGAtcacacctcttc | 69    | 0.7 | 0.1 |
| O3-mTetR  | TCCCGcTCAGTGAGACGAGAtcacacctcttcTCCCGcTCAGTGAGACGAGAtcacacctcttc | 89    | 0.7 | 0.1 |
| O1-CymR   | ACAACACAGACAACTCGTCTGTTTGTATTactcaacctatg[N_{21}]ACAA | 69    | 2    | 593 |
| O2-CymR   | [N_{21}]ACAAACACAGACAACTCGTCTGTTTGTATTacACAAACACAGACAACTCGTCTGTTTGTATTacACAAACACAGACAACTCGTCTGTTTGTATTacACAAACACAGACAACTCGTCTGTTTGTATTac | 88    | 2.3 | 270 |
| O3-CymR   | ACAACACAGACAACTCGTCTGTTTGTATTactcaacctatg[N_{21}]ACAA | 89    | 2.3 | 511 |
| O4-CymR   | ACAACACAGACAACTCGTCTGTTTGTATTactcaacctatg[N_{21}]ACAA | 107   | 2.6 | 247 |
| O1-PhlF   | [N_{21}]ATGATACGAAACGTCACCGTATCGTAAAGGTc | 630   | 2.8 | 123 |
| O2-PhlF   | [N_{21}]ATGATACGAAACGTCACCGTATCGTAAAGGTcATGATACGAAACGTCACCGTATCGTAAAGGTc | 731   | 2.9 | 0.1 |
| O3-PhlF   | ATGATACGAAACGTCACCGTATCGTAAAGGTcacaacctatg[N_{21}]ATGATACGAAACGTCACCGTATCGTAAAGGTc | 586   | 2.8 | 68 |
| O4-PhlF   | ATGATACGAAACGTCACCGTATCGTAAAGGTcacaacctatg[N_{21}]ATGATACGAAACGTCACCGTATCGTAAAGGTc | 987   | 3.9 | 0.1 |
| O1-LmrA*  | [N_{21}]GATAATAGACCAGCTTACTATATATTTtagctac | 1670  | 2.4  | 485.7 |
| O3-LmrA*  | GATAATAGACCAGCTTACTATATATTTtagctac | 1550  | 3.8  | 111.3 |
| Name     | Sequence\(^a\) | [Activator]\(/K_A\) |
|----------|----------------|---------------------|
| PEFC11w1 | TGATCC \([N_{16}]\) CGTAACACCTCTG | 0.376 \((K_A = 73,425)\) |
| PEFC11M1 | AGATCC \([N_{16}]\) CGTAACACCTCTG | 0.178 |
| PEFC11M2 | CGATCC \([N_{16}]\) CGTAACACCTCTG | 0.110 |
| PEFC11M3 | TGAGCC \([N_{16}]\) CGTAACACCTCTG | 0.0557 |
| PEFC11M4 | TGCTCC \([N_{16}]\) CGTAACACCTCTG | 0.0298 |
| PEFC11M5 | TGTTCC \([N_{16}]\) CGTAACACCTCTG | 0.0108 |

| Operators |
|----------|
| Name     | Sequence\(^c\) | \(K_R\) | \(n_R\) | \(\delta_R\) |
|----------|----------------|-------|-------|-------|
| O1-cI434 | actcttcatccggcta\([N_{13}]\)TACAAGAAAGTTTGGTcagt | 720   | 1.5   | 232   |
| O2-cI434 | actcttcatccggcta\([N_{13}]\)TACAAGAAATTTTTGTTgctacTACAAGAAAGTTTGGTcagt | 263   | 2.0   | 94    |
| O3-cI434 | TACAAGAAAGTTTGGT\([N_{13}]\)TACAAGAAAGTTTGGTcagt | 205   | 2.3   | 35    |
| O4-cI434 | TACAAGAAAGTTTGGT\([N_{13}]\)TACAAGAAATTTTTGTTgctacTACAAGAAAGTTTGGTcagt | 155   | 2.4   | 12    |

\(^a\) Mutations are shown in red.

\(^b\) Single operators are indicated by capital letters. \([N_{21}]\) indicates the random sequences to be replaced by the T7 promoter core.

\(^c\) Sequence fragments upstream of \([N_{13}]\) can be used to replace the \([N_{16}]\) region in the ECF11 promoter core; sequence fragments downstream of \([N_{13}]\) can be placed immediately downstream of the promoter core.
### Supplementary Table 4. Sequences of Crucial Parts of the pPT and pRG Backbones.

| Part Number | Function | Sequence |
|-------------|----------|----------|
| I | Terminator | `CTCGGTACAAATTCAGGAAGGCGGCAAGCCGCTTTTCTGTGTTTTGGCTCTACTATGCTCAATCATCTG` |
| II | Golden Gate marker<sup>b</sup> | `GgagaccTTACCAGTCGCTATAGCTCAGGACGGCAGCGGCTTTTCTGTGTTTTGGCTCTACTATGCTCAATCATCTG` |
| III | Ribozyme-based insulator | `AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGCCAGCTACAAATTTTGTTTAA` |
| IV | Reporter gene<sup>c</sup> | `tactagagaaaagagaaaatactagATGCGTAAAGGCGAAGAGCTGTTCACTG` |
| V | Composite terminator | `TGATAAGCCATCCAAAATAATCCAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCAAGGTGGCAGGCTTCTGCCAGACAATCAACTGCTGATTGCCCTTCACCGCCTGGCCCTGAGAGGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGGTGTTAACGGCGGGATATAACATGAGCTATCTTCGGTATCGTCGTATCCCACTACCGAGATATCCGCACCAACGCGCAGCCCGACTCGGTAATGGCGCGCATTCATTCAGCATTTGCATGGTTTGTTGAAAACCGGACATGGCACTCCAGTCGCCCTCCCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAGATATTTATGCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGACCAGATGCTCCACGCCCAGTCGCGTACCGTCCTCAGTGGAAGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGCGCGAGAAGATTGTGCACCGCGCTTTACAGGCCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGC` |
| VI | Constitutively expressed lacI<sup>d</sup> (in reverse orientation) | `TCACCTGCCGCTCTCCAGTCTGGGGAACCTGTGTGCTCCAGCTGATTATGTAATGCTCCACGCCAACGCGGCAAGCTGGTCTGATTGGGCCTTTCCGCTCGATGCCTAGTTGGTGGGATTTGGTCTGCCGCTCGTGGTGGGCTTTTCACCAGTGAGACTGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGAGAGGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGGTGTTAACGGCGGGATATAACATGAGCTATCTTCGGTATCGTCGTATCCCACTACCGAGATATCCGCACCAACGCGCAGCCCGACTCGGTAATGGCGCGCATTCATTCAGCATTTGCATGGTTTGTTGAAAACCGGACATGGCACTCCAGTCGCCCTCCCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAGATATTTATGCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGACCAGATGCTCCACGCCCAGTCGCGTACCGTCCTCAGTGGAAGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGCGCGAGAAGATTGTGCACCGCGCTTTACAGGCCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGC` |
VII Inducible promoter$^d$

| VII | Inducible promoter$^d$ |
|-----|------------------------|
| ACCCAGTTGATCGGCAGAGATTTAATCGCCGACAGATTTGCGACGCGCGTGGCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCATACGACCTGTGTTGCCCAGCCAG |
| TTCGTCAGGCCACATAGCTTTCTTGTTCTGATCGGAACGATCGTTGGCTGACAGAATTAATCATCGGCTCGTATAATGTGTGAGCGCTCACAATT |

$^a$ Neighboring parts were seamlessly ligated.

$^b$ BsaI recognition sites are indicated with lower-case letters.

$^c$ The CDS is indicated with capital letters.

$^d$ The underlined regions show the -35, -10 regions and lacO sequence of P_TAC.
| Experiment                                      | Plasmid 1 (Cmr-pSC101) | Plasmid 2 (Ampr-P15A) | Cassette Integrated into Chromosome (via pOSIP-KO) | Inducer   |
|-----------------------------------------------|------------------------|-----------------------|-----------------------------------------------------|-----------|
| Identification of insulated promoter cores    | \(^{a}\)T7 RNAP        | pRGc                  | \(^{b}\)P<sub>sal</sub>-RNAP                        | none      |
|                                               | \(^{a}\)σ<sub>ECF11</sub> |                       | \(^{c}\)P<sub>c</sub>-RNAP                         | none      |
|                                               | \(^{a}\)σ<sub>ECF16</sub> |                       |                                                     | 1 mM IPTG |
|                                               | MmP1 RNAP              |                       |                                                     | 1 mM IPTG |
|                                               | gh-1 RNAP              |                       |                                                     | 100 μM IPTG|
|                                               | T3 RNAP                |                       |                                                     | 100 μM IPTG|
| Refinement of operators                       | pPT-operator           | pRGc                  | --                                                  | none      |
| Transcriptional activation                    | [IPTG]-Input curve     | pPTc                  | pRGc                                                | P<sub>TAC</sub>-sfGFP | IPTG gradient |
|                                               | [IPTG]-Output curve    | pPT-promoter-core     | pRGc                                                | P<sub>TAC</sub>-RNAP | IPTG gradient |
| Transcriptional repression                    | [IPTG]-Input curve     | pPTc                  | pRG-sfGFP                                           | P<sub>C/TAC</sub>-RNAP | IPTG gradient |
|                                               | [IPTG]-Output curve    | pPT-promoter-operator | pRG-repressor                                      | P<sub>c</sub>-RNAP | IPTG gradient |
| IFFL                                          | pPT-promoter-operator  | pRG-repressor         | P<sub>TAC</sub>-RNAP                                | IPTG gradient |

\(^{a}\) These plasmid specifications were also used for the measurement of promoter core libraries.

\(^{b}\) In this study, P<sub>sal</sub> was used as a constitutive promoter due to its high-level basal transcriptional activity.

\(^{c}\) P<sub>c</sub>: constitutive promoter.
### Supplementary Table 6. Sequences of Cassettes integrated into the Chromosome of E. coli DH10B.

| Cassette         | Sequencea                                      |
|------------------|------------------------------------------------|
| **P*Sal-T7 RNAP** | gaattcgcgcgcgttcctagagTCAATCCGTAAACAGGTGAATACATTG CTGAGGCTTGTTGATGATGTGCTGACTGTTGCTGAGGTGAATTGCTGAGGTC   |
| **PTAC-sfGFP**   | gaattcgcgcgcgttcctagagTCAATCCGTAAACAGGTGAATACATTG CTGAGGCTTGTTGATGATGTGCTGACTGTTGCTGAGGTGAATTGCTGAGGTC   |
| **PTAC-RNAP**    | gaattcgcgcgcgttcctagagTCAATCCGTAAACAGGTGAATACATTG CTGAGGCTTGTTGATGATGTGCTGACTGTTGCTGAGGTGAATTGCTGAGGTC   |
| **P*–σECF11**   | gaattcgcgcgcgttcctagagTCAATCCGTAAACAGGTGAATACATTG CTGAGGCTTGTTGATGATGTGCTGACTGTTGCTGAGGTGAATTGCTGAGGTC   |
| **P*gh-1 RNAP**  | gaattcgcgcgcgttcctagagTCAATCCGTAAACAGGTGAATACATTG CTGAGGCTTGTTGATGATGTGCTGACTGTTGCTGAGGTGAATTGCTGAGGTC   |
| **P*–T3 RNAP**   | gaattcgcgcgcgttcctagagTCAATCCGTAAACAGGTGAATACATTG CTGAGGCTTGTTGATGATGTGCTGACTGTTGCTGAGGTGAATTGCTGAGGTC   |

a The sequences of RNAP or ECF σ immediately downstream of these sequences are omitted for clarity. The EcoRI sites are shown in bold. The underlined sequences indicate the -35 and -10 regions of the promoters.

b The CDS of NahR is shown in capital letters.

c The sequence of RiboJ is included.
Supplementary Table 7. The RBS Sequences Used in Combination with RNAP Genes in Different Cassettes.

| Cassette                  | RBS               |
|---------------------------|-------------------|
| P\textsubscript{TAC}-T7 RNAP | tactagagtatcagtaacagatactag |
| P\textsubscript{TAC}-\textsuperscript{σ}\textsubscript{ECF11} | tactagagtacacaggaaggcctcg |
| pRG-gh-1 RNAP             | Same as P\textsubscript{c}-RNAP in Supplementary Table 1 |
| pRG-T3 RNAP               | Same as P\textsubscript{c}-RNAP in Supplementary Table 1 |
| pRG-MmP1 RNAP             | As shown in Supplementary Table 1 |
| pRG-\textsuperscript{σ}\textsubscript{ECF16} | As shown in Supplementary Table 1 |
| pRG-\textsuperscript{σ}\textsubscript{ECF20} | As shown in Supplementary Table 1 |

\textsuperscript{a} These sequences correspond to the sequences in shown in lower-case letters in Supplementary Table 1.
Supplementary References

1. Rhodius, V. A. et al. Design of orthogonal genetic switches based on a crosstalk map of sigmas, anti-sigmas, and promoters. *Molecular systems biology* **9**, 702, doi:10.1038/msb.2013.58 (2013).

2. Lou, C., Stanton, B., Chen, Y. J., Munsky, B. & Voigt, C. A. Ribozyme-based insulator parts buffer synthetic circuits from genetic context. *Nature biotechnology* **30**, 1137-1142, doi:10.1038/nbt.2401 (2012).

3. Carlson, N. G. *Characterization of the repressor from the lambdoid phage HK022* (The University of Arizona, Unpublished PhD dissertation, 1992).

4. Poteete, A. R. & Ptashne, M. Operator Sequences of Bacteriophages P22 and 21. *Journal of molecular biology* **137**, 81-91 (1980).

5. Pedersen, M., Ligowska, M. & Hammer, K. Characterization of the CI repressor protein encoded by the temperate lactococcal phage TP901-1. *J Bacteriol* **192**, 2102-2110, doi:10.1128/JB.01387-09 (2010).

6. A Johnson, B. J. M. & Ptashne, M. Mechanism of action of the cro protein of bacteriophage lambda. *PNAS* **75**, 1783-1787 (1978).

7. Vershon, A. K., Liao, S.-M., McClure, W. R. & Sauer, R. T. Bacteriophage P22 Mnt repressor DNA binding and effects on transcription in vitro. *Journal of molecular biology* **195**, 311-322 (1987).

8. Stanton, B. C. et al. Genomic mining of prokaryotic repressors for orthogonal logic gates. *Nat Chem Biol* **10**, 99-105, doi:10.1038/nchembio.1411 (2014).

9. Krueger, M., Scholz, O., Wisshak, S. & Hillen, W. Engineered Tet repressors with recognition specificity for the tetO-4C5G operator variant. *Gene* **404**, 93-100, doi:10.1016/j.gene.2007.09.002 (2007).