

Supplementary Materials

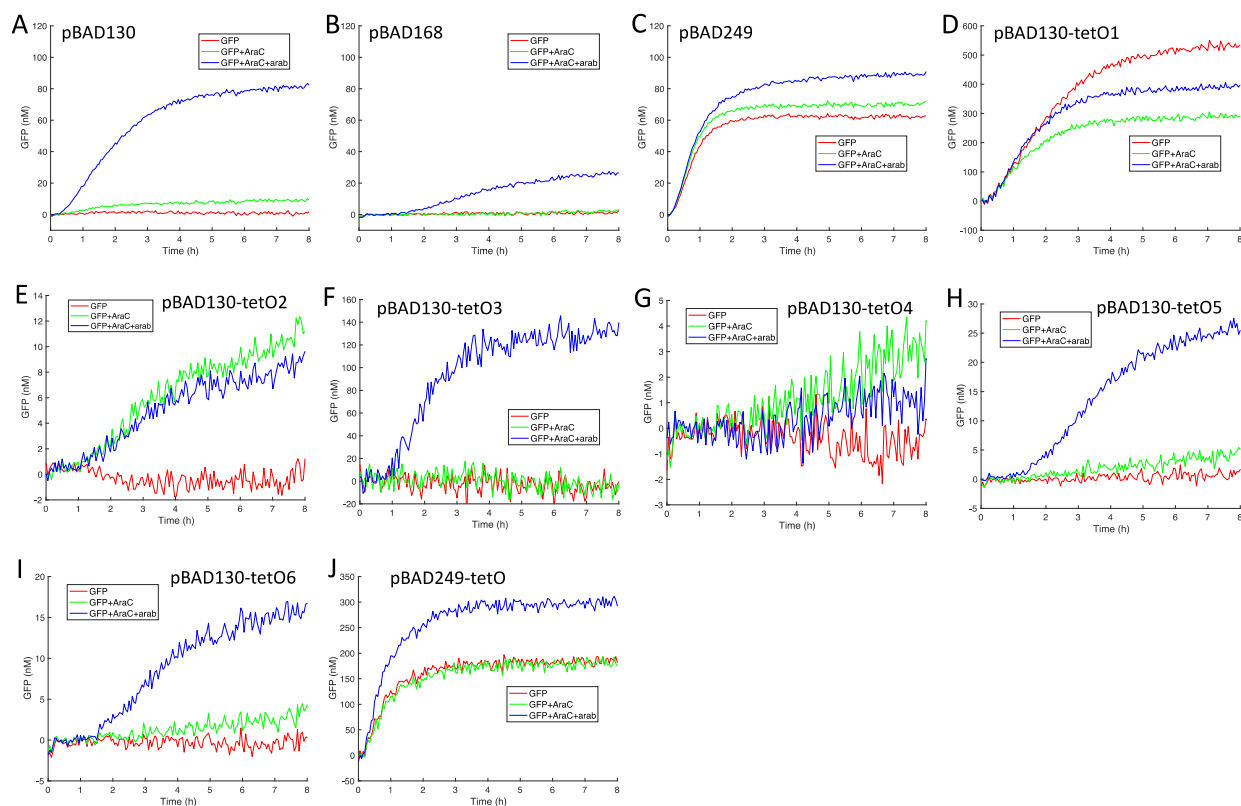


Figure S1: pBAD and pBAD-tetO promoters screening in TX-TL using linear DNAs. All experiments were run with different combinations of 10 nM pX-deGFP linear DNA (GFP), 10 nM AraC linear DNA (AraC), and 0.2% arabinose (arab) as indicated in the legends. The promoter pX used in each experiment is annotated in the corresponding subfigure.

We tested three different pBAD promoters and seven different pBAD-tetO combinatorial promoters for the AraC-TetR-deGFP FFL circuit. As we can see in Figure S1 A-C, pBAD130 (the promoter is 130 bp long) had the most desired performance, where pBAD130-deGFP alone produced little expression; and adding AraC did not change the signal significantly; only when both AraC and arabinose were present, could we see a significant expression of GFP. The activation fold-change of GFP-AraC-arab/GFP was over 50 fold. It was also clear that pBAD168 (the promoter is 168 bp long) was not as good as a promoter compared with pBAD130. The third promoter pBAD249 (the promoter is 249 bp long) had a significant expression when activated, but it did have a leaky expression where we saw GFP signal in GFP only sample. We decided to pick

pBAD130 promoter as our first choice and pBAD249 as the second candidate; pBAD168 was eliminated because of the low signal. We then used pBAD promoters as the base, and designed and tested seven pBAD-tetO combinatorial promoters. Because pBAD130 promoter was the most promising, we built six different pBAD130-tetO promoters. The construction of combinatorial promoters was based on the study in this paper.¹ We then tested these combinatorial promoters in TX-TL using linear DNAs, and the results were shown in Figure S1 D-J. Promoters pBAD130-tetO1 and 4 (Figure S1 D, G) were immediately eliminated based on the expression curves as they did not show significant activation at all. Among the other 4 pBAD130-tetO, pBAD130-tetO3 stood out of the candidates based on its large activation fold-change and little expression when AraC was added. pBAD249-tetO had similar expression profile compared to the pBAD249 promoter that it was based on. Then two versions of the AraC-TetR-deGFP FFL circuit was eventually built and tested extensively in TX-TL (with linear DNAs) and in cells: A. pBAD130-TetR and pBAD130-tetO-deGFPssrA; B. pBAD249-TetR and pBAD249-tetO-deGFPssrA. The results shown in the main text Figure 1 were all from the version A FFL circuit. Results from the version B FFL circuit were shown in Figure S2.

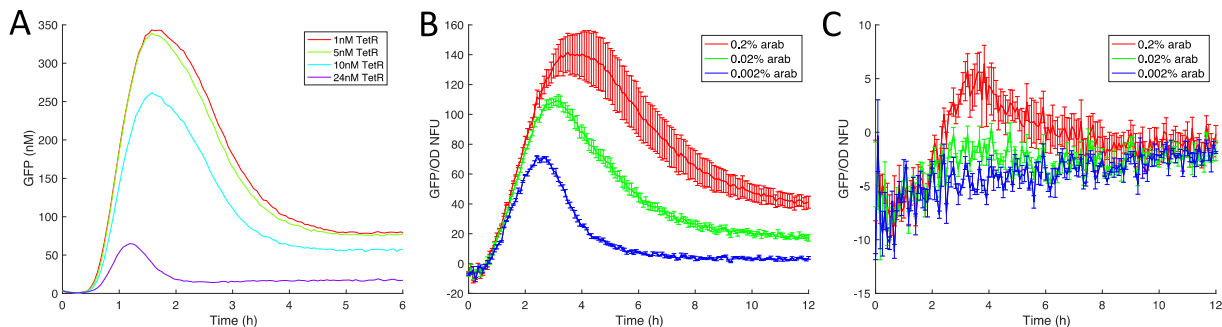


Figure S2: Test the version B AraC-TetR-deGFP FFL circuit in TX-TL and cells. **A:** Experimental results of the FFL in TX-TL with 10 nM AraC linear DNA, 10 nM deGFPssrA linear DNA, 10 nM ClpX linear DNA, 0.2% arabinose, 100 ng/mL aTc and varied TetR linear DNA concentrations. **B, C:** The time course results of the FFL circuit in cells induced with a range of arabinose concentrations. The deGFP protein is tagged with the LAA-ssrA degradation tag. In **B**, 1 ng/mL of aTc was added, and there was no aTc in **C**. For both **B** and **C**, data were averaged from three independent repeated wells and then were normalized using OD600 readings to get the fluorescence reading for each cell (normalized fluorescence unit, NFU). Error bars represent one standard deviation from three independent experiments.

As we can see in Figure S2 A-B, the version B FFL circuit showed pulse-like behavior in both TX-TL and cells. Besides, when we decreased TetR DNA concentrations or increased arabinose concentrations, we observed higher peaks in the pulses. However, there was one extra component added in these experiments to achieve this behavior, which was anhydrotetracycline (aTc). As we mentioned in the main text, TetR proteins can bind to the tetO part in the pBAD-tetO promoter and stop the transcription of the downstream gene. In the presence of aTc, free TetR proteins will first bind to aTc because of the higher binding affinity. Only after all the free aTc was bound by TetR, the pBAD-tetO promoter would be occupied by TetR and ceased the transcription. Because pBAD249 promoter had a leaky expression, we had to add aTc to the reaction to extend the time of transcription for the deGFP gene before it got repressed by TetR. Although it was not optimal, we could still get pulses with the help of aTc, as shown in Figure S2 A-B. As a comparison, aTc was not added to the experiment in Figure S2C, and we only saw little expression of GFP at the highest arabinose concentration (and a small pulse as well). In conclusion, in both versions of the FFL circuits, we were able to translate the results in TX-TL to cells, and both versions were indeed feedforward loops both *in vitro* and *in vivo*.

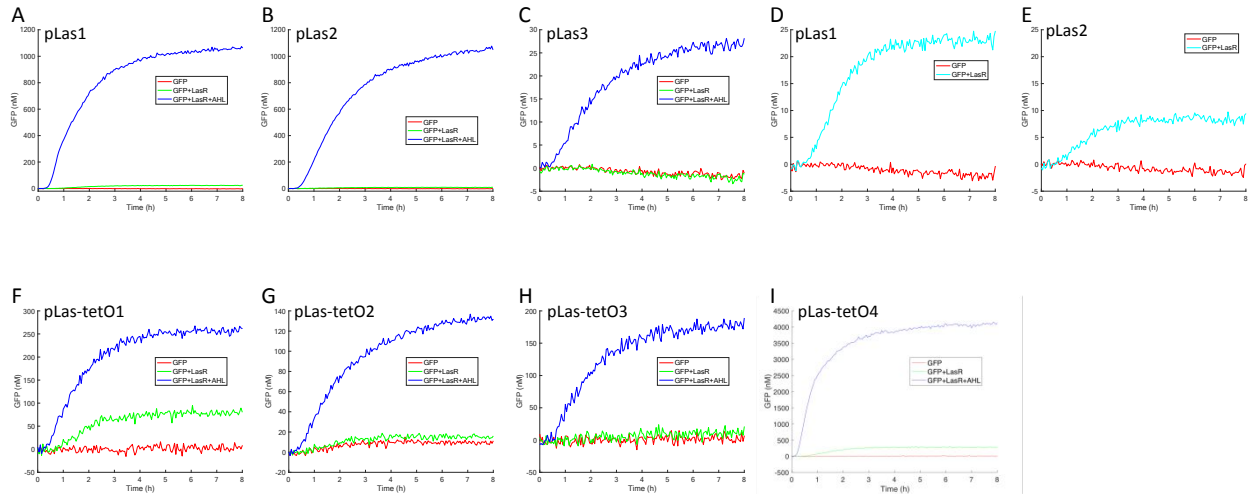


Figure S3: pLas and pLas-tetO promoters screening in TX-TL using linear DNAs. All experiments were run with different combinations of 10 nM pX-deGFP linear DNA (GFP), 10 nM LasR linear DNA (LasR), and 100 nM AHL (AHL) as indicated in the legends. The promoter pX used in each experiment is annotated in the corresponding subfigure.

We tested three different pLas promoters and four different pLas-tetO promoters in TX-TL using linear DNAs. As we can see in Figure S3 A-C, both pLas1 and pLas2 had great activation when both LasR and AHL were added. Although a zoom-in look at both Figure S3 A and B showed that pLas1 had a bit more expression when LasR was added without AHL (Figure S3 D and E). While Figure S3 F-I suggested that pLas-tetO2 and pLas-tetO3 could be good candidates for the combinatorial promoter. Both pLas-tetO1 and pLas-tetO4 had significant expression when LasR was added without AHL, while the much higher signal from pLas-tetO4 might not be a positive feature when applied in cells. As we showed in Figure S4A, cells with pLas-tetO4 showed no expression at all. This could be a result of too much stress on cells. Based on these results, we picked pLas2 and pLas-tetO3 as our top choices (the best version, results shown in main text Figure 2B), and pLas1 and pLas-tetO2 as the follow-up version (Figure S4 B-D). The follow-up version FFL showed little activation at 0 aTc concentration (Figure S4B). As we increased aTc concentrations, we saw higher expression from this FFL (Figure S4 C-D), where increasing AHL concentrations also resulted in higher activation level and more significant pulses.

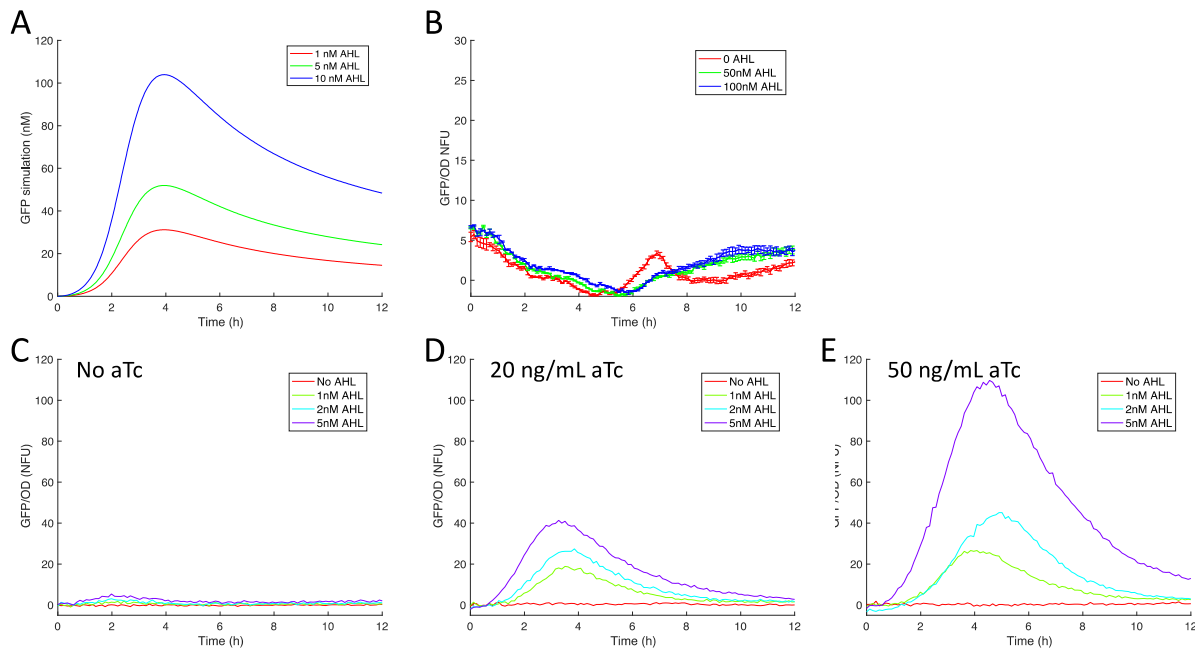


Figure S4: Test two different versions of LasR-TetR-deGFP FFL circuit in cells. The deGFP protein is tagged with the LAA-ssrA degradation tag. **A:** A simulated time course of an FFL using the TX-TL toolbox. Three curves are deGFP protein concentrations over time with increasing AHL concentrations at 1nM, 5nM, and 10nM. **B:** The time course results of a LasR-TetR-deGFP FFL circuit using pLas-tetO4-deGFP in cells induced with a range of AHL concentrations. **C, D, E:** The time course results of the follow-up FFL circuit in cells induced with a range of AHL concentrations. In **C**, no aTc was added, and in **D** and **E**, 20 ng/mL or 50 ng/mL of aTc was used (indicated in the plot). Data were subtracted by the background and normalized using OD600 readings to get the fluorescence reading for each cell (normalized fluorescence unit, NFU).

DNA Sequences of the promoters and coding sequences used in this work:

1. pBAD130:

ACATTGATTATTTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCC
ATAAGATTAGCGGATCCTACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCT
CCATACCGTTTTTTTTGGGCTAGC

2. pBAD168:

GCGTAACAAAAGTGTCTATAATCACGGCAGAAAAGTCCACATTGATTATTTG
CACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCCATAAGATTAGCGGA
TCCTACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCGTTTTTT
GGGCTAGC

3. pBAD249:

AGAAACCAATTGTCCATATTGCATCAGACATTGCCGTCCTGCGTCTTTTACT
GGCTCTTCTCGCTAACCAAACCGGTAACCCCGCTTATTAAGCATTCTGTAA
CAAAGCGGGACCAAAGCCATGACAAAAACGCGTAACAAAAGTGTCTATAAT
CACGGCAGAAAAGTCCACATTGATTATTTGCACGGCGTCACACTTTGCTATGC
CATAGCATTTTTATCCATAAGATTAGCGGATCCTACCTG

4. pBAD130-tetO1:

ACATTGATTATTTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCC
ATAAGATTAGCGGATCCTACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCT
CCATACCGTTTTTTTTGGGCTAGCTCCCTATCAGTGATAGAGATTGACATCCCT
ATCAGTGATAGAGATACTGAGCACA

5. pBAD130-tetO2:

ACATTGATTATTTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCC
ATAAGATTAGCGGATCCTACCTGATCCCTATCAGTGATAGAGATCGCTTTTTA
TCGCAACTCTCTACTGTTTCTCCATACCGTTTTTTTTGGGCTAGC

6. pBAD130-tetO3:

ACATTGATTATTTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCC
ATAAGATTAGCGGATCCTACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCT
CCATACCGTTTTTTTTGGGCTAGCTCCCTATCAGTGATAGAGAT

7. pBAD130-tetO4:

ACATTGATTATTTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCC
ATAAGATTAGCGGATCCTCCCTATCAGTGATAGAGATTACCTGACGCTTTTAA
TCGCAACTCTCTACTGTTTCTCCATACCGTTTTTTTTGGGCTAGC

8. pBAD130-tetO5:

ACATTGATTATTTGCACGGCGTCACACTTTGTCCCTATCAGTGATAGAGATCT
ATGCCATAGCATTTTTATCCATAAGATTAGCGGATCCTACCTGACGCTTTTAA
TCGCAACTCTCTACTGTTTCTCCATACCGTTTTTTTTGGGCTAGC

9. pBAD130-tetO6:

ACATTGATTATTTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCC
ATAAGATTAGCGGATCCTACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCT
CCATTCCCTATCAGTGATAGAGATAACCGTTTTTTTTGGGCTAGC

10. pBAD249-tetO:

AGAAACCAATTGTCCATATTGCATCAGACATTGCCGTCCTACTGCGTCTTTTACT
GGCTCTTCTCGCTAACCAAACCGGTAACCCCGCTTATTAAGCATTCTGTAA
CAAAGCGGGACCAAAGCCATGACAAAAACGCGTAACAAAAGTGTCTATAAT
CACGGCAGAAAAGTCCACATTGATTATTTGCACGGCGTCACACTTTGCTATGC
CATAGCATTTTTATCCATAAGATTAGCGGATCCTACCTGTCCCTATCAGTGAT
AGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACA

11. pLas1:

AAGGGTCCGGGTTACCGAAATCTATCTCATTGCTAGTTATAAAATTATGAA
ATTTGCGTAAATTCTTC

12. pLas2:

TGTTCTCGTGTGAAGCCATTGCTCTGATCTTTTGGACGTTTCTTCGAGCCTAGC
AAGGGTCCGGGTTACCGAAATCTATCTCATTGCTAGTTATAAAATTATGAA
ATTTGTATAAATTCTTCAG

13. pLas3:

GCCCCTCGCTGAGCGCGTCCCGGAGCTGGGGGCAACCTAGCTGCCACCTGCT
TTTCTGCTAGCTATTCCAGCGAAAACATACAGATTTCCGGCGAAATCAAGGCT
ACCTGCCAGTTCTGGCAGGTTTGGCCGCGGGTTCTTTTTGGTACACGA

14. pLas-tetO1:

AACTAGCAAATGAGATAGATTTCCGGTGAACCCGGACCCTTGCTAGGCTCGAT

CCCTATCAGTGATAGAGA

15. pLas-tetO2:

TTCGAGCCTAGCAAGGGTCCGGGTTACCGAAATCTATCTCATTTGCTAGTTA
TAAAATTATGAAATTTGCGTAAATTCCTATCAGTGATAGAGATTCAG

16. pLas-tetO3:

TTCGAGCCTAGCAAGGGTCCGGGTTACCGAAATCTATCTCATTTGCTAGTTA
TAAAATCCCTATCAGTGATAGAGATTATGAAATTTGCGTAAATTCCTATCAG
TGATAGAGATTCAG

17. pLas-tetO4:

GCCCCTCGCTGAGCGCGTCCCGGAGCTGGGGGCAACCTAGCTGCCACCTGCT
TTTCTGCTAGCTATTCCAGCGAAAACATACAGATTTCCGGCGAAATCAAGGCT
ACCTGCCAGTTCTGGCAGGTTTGGCCGCGGGTTCTTTTTGGTACACTCCCTAT
CAGTGATAGAG

18. deGFP:

ATGGAGCTTTTCACTGGCGTTGTTCCCATCCTGGTCGAGCTGGACGGCGACGT
AAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTA
CGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCCGTGCC
TGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTA
CCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGC
TACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCC
GCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGA
AGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGT
ACAACACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACG
GCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCA
GCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTG
CTGCCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCA
ACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCCGGGAT
C

19. LAA-ssrA degradation tag: GCAGCAAACGACGAAAACACTACGCTTTAGCTGCT

20. TetR:

ATGTCTAGATTAGATAAAAAGTAAAGTGATTAACAGCGCATTAGAGCTGCTTA

ATGAGGTCGGAATCGAAGGTTTAACAACCCGTAAACTCGCCCAGAAGCTAGG
TG TAGAGCAGCCTACATTGTATTGGCATGTAAAAAATAAGCGGGCTTTGCTC
GACGCCTTAGCCATTGAGATGTTAGATAGGCACCATACTCACTTTTGCCCTTT
AGAAGGGGAAAGCTGGCAAGATTTTTTACGTAATAACGCTAAAAGTTTTAGA
TGTGCTTTACTAAGTCATCGCGATGGAGCAAAGTACATTTAGGTACACGGC
CTACAGAAAACAGTATGAAACTCTCGAAAATCAATTAGCCTTTTTATGCCA
ACAAGGTTTTTCACTAGAGAATGCATTATATGCACTCAGCGCTGTGGGGCATT
TTACTTTAGGTTGCGTATTGGAAGATCAAGAGCATCAAGTCGCTAAAGAAGA
AAGGGAAACACCTACTACTGATAGTATGCCGCCATTATTACGACAAGCTATC
GAATTATTTGATCACCAAGGTGCAGAGCCAGCCTTCTTATTCGGCCTTGAATT
GATCATATGCGGATTAGAAAAACA ACTTAAATGTGAAAGTGGGTCT

21. AraC:

ATGCAATATGGACAATTGGTTTCTTCTCTGAATGGCGGGAGTATGAAAAGTA
TGGCTGAAGCGCAAATGATCCCCTGCTGCCGGGATACTCGTTTAATGCCCAT
CTGGTGGCGGGTTTAACGCCGATTGAGGCCAACGGTTATCTCGATTTTTTTAT
CGACCGACCGCTGGGAATGAAAGGTTATATTCTCAATCTCACCATTCGCGGGT
AGGGGGTGGTGAAAAATCAGGGACGAGAATTTGTTTGCCGACCGGGTGATAT
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CAAGGGGAAGGGCGCTATTCGGAGCTGCTGGCGATAAATCTGCTTGAGCAAT
TGTTACTGCGGCGCATGGAAGCGATTAACGAGTCGCTCCATCCACCGATGGA
TAATCGGGTACGCGAGGCTTGTCAGTACATCAGCGATCACCTGGCAGACAGC
AATTTTGATATCGCCAGCGTCGCACAGCATGTTTGCTTGTCGCCGTCGCGTCT
GTCACATCTTTTCCGCCAGCAGTTAGGGATTAGCGTCTTAAGCTGGCGCGAGG
ACCAACGTATCAGCCAGGCGAAGCTGCTTTTGAGCACCACCCGGATGCCTAT
CGCCACCGTCGGTCGCAATGTTGGTTTTGACGATCAACTCTATTTCTCGCGGG
TATTTAAAAAATGCACCGGGGCCAGCCCGAGCGAGTCCGTGCCGGTTGTGA
AGAAAAAGTGAATGATGTAGCCGTCAAGTTGTCA

22. LasR:

ATGGCCTTGGTTGACGGTTTTCTTGAGCTGGAACGCTCAAGTGGAAAATTGG
AGTGGAGCGCCATCCTCCAGAAGATGGCGAGCGACCTTGGATTCTCGAAGAT
CCTGTTCGGCCTGTTGCCTAAGGACAGCCAGGACTACGAGAACGCCTTCATC
GTCGGCAACTACCCGGCCGCCTGGCGCGAGCATTACGACCCGGGCTGGCTACG
CGCGGGTCGACCCGACGGTCAGTCACTGTACCCAGAGCGTACTGCCGATTTT
CTGGGAACCGTCCATCTACCAGACGCGAAAGCAGCACGAGTTCTTCGAGGAA
GCCTCGGCCGCCGGCCTGGTGTATGGGCTGACCATGCCGCTGCATGGTGCTC
GCGGCGAACCTCGGCGCGCTGAGCCTCAGCGTGGAAGCGGAAAACCGGGCCG
AGGCCAACCGTTTCATAGAGTCGGTCCTGCCGACCCTGTGGATGCTCAAGGA
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GAACTTCCATATGGGAAATATTCGGCGGAAGTTCGGTGTGACCTCCCGCCGC
GTAGCGGCCATTATGGCCGTTAATTTGGGTCTTATTACTCTCTAA

References

1. Cox, R. S., 3rd; Surette, M. G.; Elowitz, M. B., Programming gene expression with combinatorial promoters. *Mol Syst Biol* **2007**, *3*, 145.