Supplementary Materials

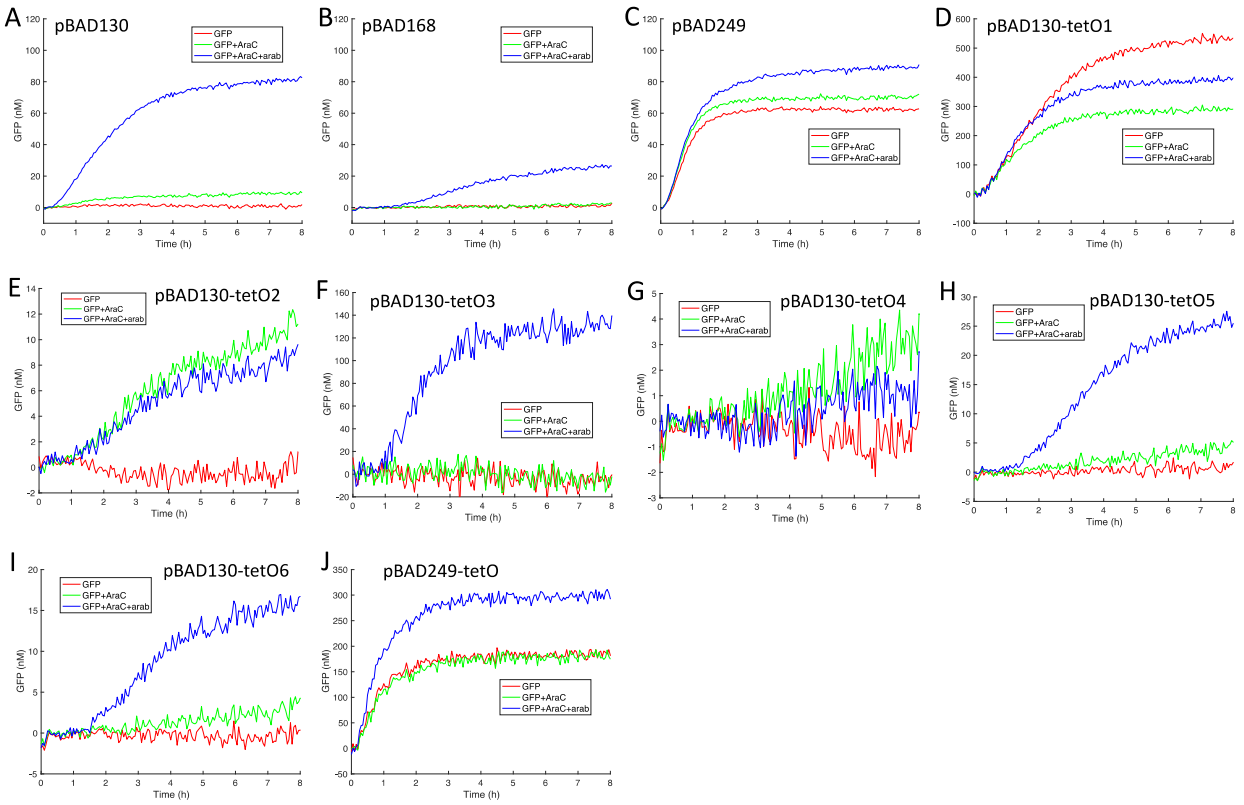

Figure S1: pBAD and pBAD-tetO promoters screening in TX-TL using linear DNAs. All experiments were run with different combinations of 10 nM pX-deGFP linear DNA (GFP), 10 nM AraC linear DNA (AraC), and 0.2% arabinose (arab) as indicated in the legends. The promoter pX used in each experiment is annotated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

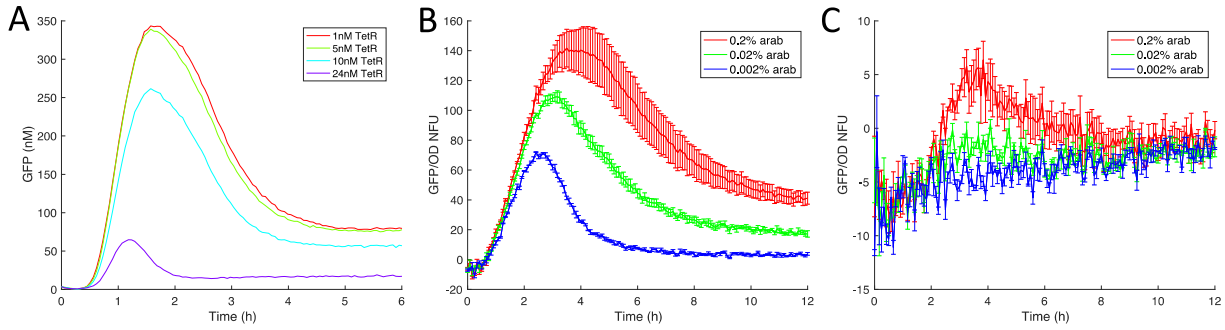

Figure S2: Test the version B AraC-TetR-deGFP FFL circuit in TX-TL and cells. **A:** Experimental results of the FFL in TX-TL with 10 nM AraC linear DNA, 10 nM deGFPssrA linear DNA, 10 nM ClpX linear DNA, 0.2% arabinose, 100 ng/mL aTc and varied TetR linear DNA concentrations. **B, C:** The time course 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*.

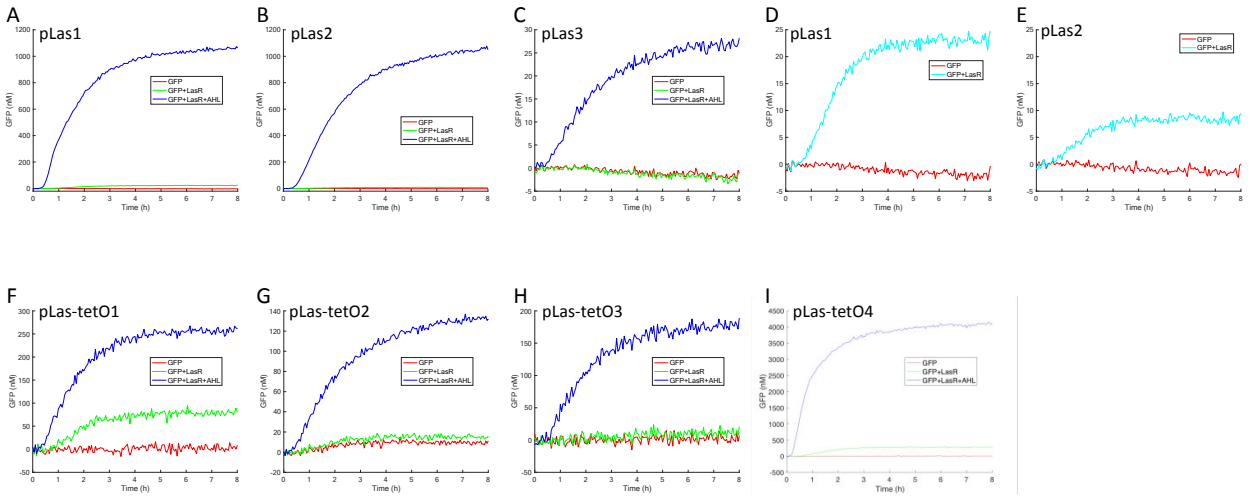

Figure S3: pLas and pLas-tetO promoters screening in TX-TL using linear DNAs. All experiments were run with different combinations of 10 nM pX-deGFP linear DNA (GFP), 10 nM LasR linear DNA (LasR), and 100 nM AHL (AHL) as indicated in the legends. The promoter pX used in each experiment is annotated in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

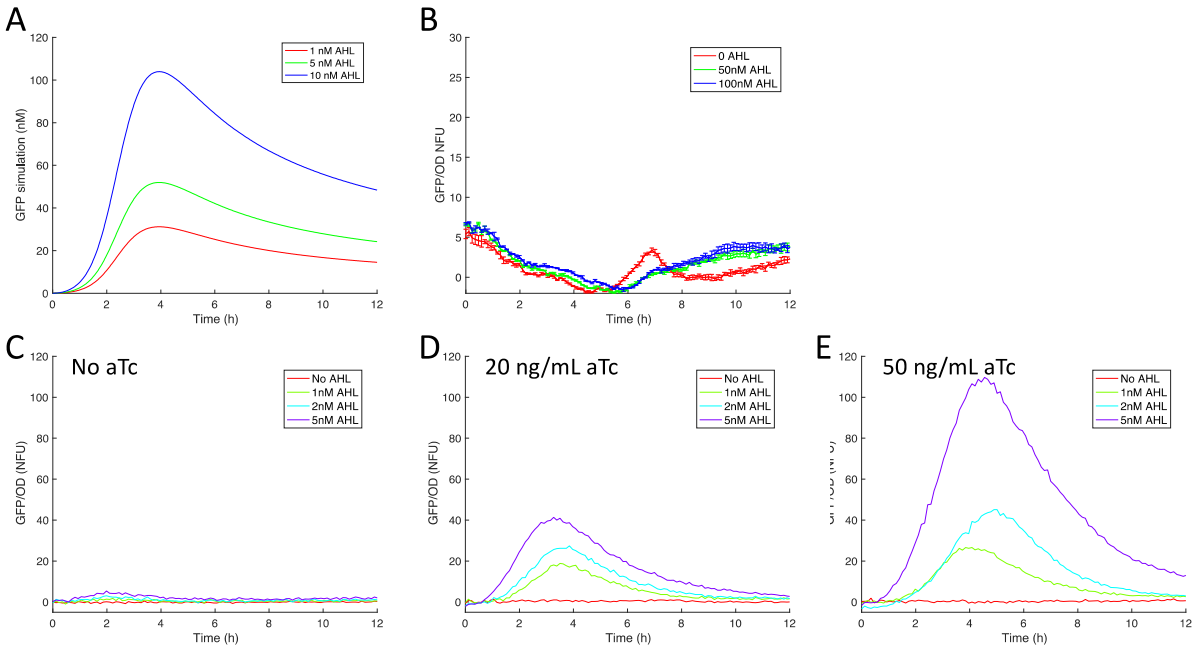

Figure S4: Test two different versions of LasR-TetR-deGFP FFL circuit in cells. The deGFP protein is tagged with the LAA-ssrA degradation tag. A: A simulated time course of an FFL using the TX-TL toolbox. Three curves are deGFP protein concentrations over time with increasing AHL concentrations at 1nM, 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
   CCATACCGTTTTTTTGGGCTAGC
2. pBAD168:
   GCGTAACAAAAGTGTCTATAATCACGGCAGAAAAGTCCACATTGATTATTTG
   CACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCCATAAGATTAGCGGA
   TCCTACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCGTTTTTTT
   GGGCTAGC
3. pBAD249:
   AGAAACCAATTGTCCATATTGCATCAGACATTGCCGTCACTGCGTCTTTTACT
   GGCTCTTCTCGCTAACCAAACCGGTAACCCCGCTTATTAAAAGCATTCTGTAA
   CAAAGCGGGACCAAAGCCATGACAAAAACGCGTAACAAAAGTGTCTATAAT
   CACGGCAGAAAAGTCCACATTGATTATTTGCACGGCGTCACACTTTGCTATGC
   CATAGCATTTTTATCCATAAGATTAGCGGATCCTACCTG
4. pBAD130-tetO1:
   ACATTGATTATTTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCC
   ATAAGATTAGCGGATCCTACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCT
   CCATACCGTTTTTTTGGGCTAGCTCCCTATCAGTGATAGAGATTGACATCCCT
   ATCAGTGATAGAGATACTGAGCACA
5. pBAD130-tetO2:
   ACATTGATTATTTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCC
   ATAAGATTAGCGGATCCTACCTGATCCCTATCAGTGATAGAGATCGCTTTTTA
   TCGCAACTCTCTACTGTTTCTCCATACCGTTTTTTTGGGCTAGC
6. pBAD130-tetO3:
   ACATTGATTATTTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCC
   ATAAGATTAGCGGATCCTACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCT
   CCATACCGTTTTTTTGGGCTAGCTCCCTATCAGTGATAGAGAT
7. pBAD130-tetO4:

ACATTGATTATTTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCC
ATAAGATTAGCGGATCCTCCCTATCAGTGATAGAGATTACCTGACGCTTTTTA
TCGCAACTCTCTACTGTTTCTCCATACCGTTTTTTTGGGCTAGC

8. pBAD130-tetO5:
   ACATTGATTATTTGCACGGCGTCACACTTTGTCCCTATCAGTGATAGAGATCT
   ATGCCATAGCATTTTTATCCATAAGATTAGCGGATCCTACCTGACGCTTTTTA
   TCGCAACTCTCTACTGTTTCTCCATACCGTTTTTTTGGGCTAGC
9. pBAD130-tetO6:
   ACATTGATTATTTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCC
   ATAAGATTAGCGGATCCTACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCT
   CCATTCCCTATCAGTGATAGAGATACCGTTTTTTTGGGCTAGC
10. pBAD249-tetO:
    AGAAACCAATTGTCCATATTGCATCAGACATTGCCGTCACTGCGTCTTTTACT
    GGCTCTTCTCGCTAACCAAACCGGTAACCCCGCTTATTAAAAGCATTCTGTAA
    CAAAGCGGGACCAAAGCCATGACAAAAACGCGTAACAAAAGTGTCTATAAT
    CACGGCAGAAAAGTCCACATTGATTATTTGCACGGCGTCACACTTTGCTATGC
    CATAGCATTTTTATCCATAAGATTAGCGGATCCTACCTGTCCCTATCAGTGAT
    AGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACA
11. pLas1:
    AAGGGTCCGGGTTCACCGAAATCTATCTCATTTGCTAGTTATAAAATTATGAA
    ATTTGCGTAAATTCTTC
12. pLas2:
    TGTTCTCGTGTGAAGCCATTGCTCTGATCTTTTGGACGTTTCTTCGAGCCTAGC
    AAGGGTCCGGGTTCACCGAAATCTATCTCATTTGCTAGTTATAAAATTATGAA
    ATTTGTATAAATTCTTCAG
13. pLas3:
    GCCCCTCGCTGAGCGCGTCCCGGAGCTGGGGGCAACCTAGCTGCCACCTGCT
    TTTCTGCTAGCTATTCCAGCGAAAACATACAGATTTCCGGCGAAATCAAGGCT
    ACCTGCCAGTTCTGGCAGGTTTGGCCGCGGGTTCTTTTTGGTACACGA
14. pLas-tetO1:
    AACTAGCAAATGAGATAGATTTCGGTGAACCCGGACCCTTGCTAGGCTCGAT

CCCTATCAGTGATAGAGA

15. pLas-tetO2:
    TTCGAGCCTAGCAAGGGTCCGGGTTCACCGAAATCTATCTCATTTGCTAGTTA
    TAAAATTATGAAATTTGCGTAAATTCCCTATCAGTGATAGAGATTCAG
16. pLas-tetO3:
    TTCGAGCCTAGCAAGGGTCCGGGTTCACCGAAATCTATCTCATTTGCTAGTTA
    TAAAATCCCTATCAGTGATAGAGATTATGAAATTTGCGTAAATTCCCTATCAG
    TGATAGAGATTCAG
17. pLas-tetO4:
    GCCCCTCGCTGAGCGCGTCCCGGAGCTGGGGGCAACCTAGCTGCCACCTGCT
    TTTCTGCTAGCTATTCCAGCGAAAACATACAGATTTCCGGCGAAATCAAGGCT
    ACCTGCCAGTTCTGGCAGGTTTGGCCGCGGGTTCTTTTTGGTACACTCCCTAT
    CAGTGATAGAG
18. deGFP:
    ATGGAGCTTTTCACTGGCGTTGTTCCCATCCTGGTCGAGCTGGACGGCGACGT
    AAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTA
    CGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCC
    TGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTA
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    GCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGA
    AGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGT
    ACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACG
    GCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCA
    GCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTG
    CTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCA
    ACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGAT
    C
19. LAA-ssrA degradation tag: GCAGCAAACGACGAAAACTACGCTTTAGCTGCT
20. TetR:
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ATGAGGTCGGAATCGAAGGTTTAACAACCCGTAAACTCGCCCAGAAGCTAGG
TGTAGAGCAGCCTACATTGTATTGGCATGTAAAAAATAAGCGGGCTTTGCTC
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21. AraC:

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TGGCTGAAGCGCAAAATGATCCCCTGCTGCCGGGATACTCGTTTAATGCCCAT
CTGGTGGCGGGTTTAACGCCGATTGAGGCCAACGGTTATCTCGATTTTTTTAT
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TAATCGGGTACGCGAGGCTTGTCAGTACATCAGCGATCACCTGGCAGACAGC
AATTTTGATATCGCCAGCGTCGCACAGCATGTTTGCTTGTCGCCGTCGCGTCT
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CGCCACCGTCGGTCGCAATGTTGGTTTTGACGATCAACTCTATTTCTCGCGGG
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AGAAAAAGTGAATGATGTAGCCGTCAAGTTGTCA

22. LasR:

ATGGCCTTGGTTGACGGTTTTCTTGAGCTGGAACGCTCAAGTGGAAAATTGG
AGTGGAGCGCCATCCTCCAGAAGATGGCGAGCGACCTTGGATTCTCGAAGAT
CCTGTTCGGCCTGTTGCCTAAGGACAGCCAGGACTACGAGAACGCCTTCATC
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GCCTCGGCCGCCGGCCTGGTGTATGGGCTGACCATGCCGCTGCATGGTGCTC
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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.