**METHODS**

* Library construction (cloning)

Each region of the riboswitch was surveyed with a library that randomized the nucleotides of interest. Libraries were constructed through PCR reaction with combined outer primers, 3’GEN-Gblock\_HindIII, 5’GEN-Gblock\_NsiI and an adaptor that spanned from the SpeI cut site to the unique HindIII cut site with a library specific inner primer. Libraries were amplified with 12-20 round of amplification. PCR products were cleaned with Omega CyclePure kit, digested with NsiI and HindIII, again cleaned with the Omega CyclePure kit, and PNK treated to improve transformation efficiency.

The vector was pRR5\_gfpUV which contains AmpR, BLAH BLAH promoter, gfpUV as a reporter protein, and a unique HindIII and NsiI cut site upstream of the fluorescent protein. The vector was digested with HindIII and NsiI and treated with cow intestinal alkaline phosphatase. The vector was then ligated with the PNK treated insert to create a construct with Amp resistance, and a gene (gfpUV) under genetic control of an upstream riboswitch.

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| --- | --- |
| Primer Name | Sequence (5` to 3`) |
| Del27\_NH5\_lib\_loop | TTTACGGGCATGCATAAGGCTCGTATAATATATTCCACTTGT  ATAACCTCAATAATATGGTTTGAGGGTGTCTACCAGGAACCG  TAAAATCCTGATTACAAGCCGNNNNNNCGGCTTGTAATCAGG  ATTTTTTTTATTTACTAGTACATTTAAGTAAAGGAGTT |
| Del27\_NH5\_lib\_stem | TTTACGGGCATGCATAAGGCTCGTATAATATATTCCACTTGT  ATAACCTCAATAATATGGTTTGAGGGTGTCTACCAGGAACCG  TAAAATCCTGATTACAANNNGTTTTTTCNNNTTGTAATCAGG  ATTTTTTTTATTTACTAGTACATTTAAGTAAAGGAGTT |
| Del27\_NH5\_lib\_gateway | TTTACGGGCATGCATAAGGCTCGTATAATATATTCCACTTGT  ATAACCTCAATAATATGGTTTGAGGGTGTCTACCAGGAACCG  TAAAATCCTGATTACAAGCCGTTTTTTCGGCTTGNNNNNNGG  ATTTTTTTTATTTACTAGTACATTTAAGTAAAGGAGTT |
| Del27\_NH5\_P4\_anchor | TTTACGGGCATGCATAAGGCTCGTATAATATATTCCACTTGT  ATAACCTCAATAATATGGTTTGAGGGTGTCTACCAGGAACCG  TAAAATCCTGATTACAAGCCGTTTTTTCNNNNNGTAATCAGG  ATTTTTTTTATTTACTAGTACATTTAAGTAAAGGAGTT |
| P1 gateway/anchor exchange |  |
| P1 exchange |  |
| P3 |  |
| 3’\_SpeI\_HindIII\_pRRadaptor | GCATGCAAGCTTGGCGTAATCATGGTCATAACAAACTCCTTT  ACTTAAATGTACTAGTA |
| 5’GEN-Gblock\_NsiI | TTTACGGGCATGCATAAGGCTCGTATA |
| 3’GEN-Gblock\_HindIII | AGGCATGCAAGCTTGGCGTAATCATGG |
| pRR\_Forward | GCGCTAGCCACAGCTAACAC |

* **Primary plating**

Following cloning, libraries were transformed into BW### Keio parental cells and plated onto minimal media plates supplemented with carbenicillin. Those libraries that destabilized the aptamer domain (XXX) were plated on medium supplemented with 0.5 mM 2AP to probe ability to express. Those that destabilized the expression platform (XXX) were plated onto unliganded plates.

Primary plates were illuminated at 3XX nm to visualize gfpUV expression. Those colonies that appeared to behave in accordance with the ligand state of the plate (dim on unliganded plates, bright on liganded plates) were selected and transferred to a set of gridded plates, one with 0.5 mM 2AP and one without ligand. Those plates were incubated overnight at 37 °C.

Colonies were again evaluated based on expression when illuminated by 3XX nm light. Those colonies that exhibited differential expression on the two plates were then moved onto a liquid activity assay and the plasmids were isolated with a Omega Plasmid Mini Prep kit and sent for sequencing by Sanger sequencing.

* **Calculations and analysis**
* **Design of specific mutants**