**Materials and Methods**

**Library construction and Colony Screening**

A genetic screen was conducted utilizing the method described in Polaski et al. 2018. The oligonucleotides used to amplify the libraries for this screen are listed in Table 1, as are the outer primers which include the single-stranded outer Ultramers which include the 3’ NsiI cut site and 5’ HindIII cut site. The same reporter plasmids with mNeon and the ampicillian resistance cassette was used and transformed into the same cell strain, though those cells were heat shocked at 37 °C for 90 s during transformation. In this screen each colony was also subjected to primary plating, quantified with Opencfu software package (3.9.0) (GEISSMAN et al, PLoS One 2013), secondary screening on gridded plates, and sequence verification.

**Reporter based Activity Assays**

Winning colonies from the gridded plates were picked and used for 3mL overnights in chemically defined salt broth (CSB) media with 100 ug/mL ampicillin. Those overnights were used in the same fashion as in the previous work to quantify switch activity with the same pBR327 negative control.

**Data Processing and Phylogenetic Tree Construction**

Activity values collected for each library were analyzed in R. Those values, for repression, expression, and fold repression, which were outside of 1.5 times the interquartile range were removed. Each colony had at least 9 separate measurements due to biological and technical triplicate. This decision was made assuming that the distribution of the data was Gaussian (https://link.springer.com/chapter/10.1007/978-981-10-7563-6\_53). Those switches with activity less than 2.0 were also removed as they did not have robust enough switching ability. For each switch, the median fluorescence with and without cyanocobalamin and median fold repression was reported. The fluoresce values were accompanied by standard error values.

Three FASTA files for each library were created with the sequence of each unique functional riboswitch ranked by fold repression, repression in the absence of cyanocobalamin, or *env8* corrected fold repression. Each library has a unique *env8* value, measured during each assay date, which is used to normalize values within each library to compare function between libraries.