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An *in vitro* selection for small molecule induced switching RNA molecules

Laura Martini [a], Andrew D. Ellington [b,*], Sheref S. Mansy [a,*]

[a] CIBIO, University of Trento, Via Sommarive 9, 38123 Povo, Italy
[b] Department of Chemistry and Biochemistry, Institute for Cellular and Molecular Biology, Center for Systems and Synthetic Biology, University of Texas at Austin, Austin, TX 78712, USA

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ABSTRACT

The selection of RNA and DNA aptamers now has a long history. However, the ability to directly select for conformational changes upon ligand binding has remained elusive. These difficulties have stymied attempts at making small molecule responsive strand displacement circuitry as well as synthetic riboswitches. Herein we present a detailed strand displacement based selection protocol to directly select for RNA molecules with switching activity. The library was based on a previously selected thiamine pyrophosphate riboswitch. The fully *in vitro* methodology gave sequences that showed strong strand displacement activity in the presence of thiamine pyrophosphate. Further, the selected sequences possessed riboswitch activity similar to that of natural riboswitches. The presented methodology should aid in the design of more complex, environmentally responsive strand displacement circuitry and in the selection of riboswitches responsive to toxic ligands.

Contents

* Corresponding authors.
E-mail addresses: ellingtonlab@gmail.com (A.D. Ellington), sheref.mansy@unitn.it (S.S. Mansy).

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1. Introduction

Nucleic acid sequences can be designed to fold into desired shapes [1] and to specifically hybridize with target sequences through simple base-pairing rules. In addition to forming static structures, the system can be dynamic in the sense that an oligonucleotide can be designed to displace one strand of a duplex potentially triggering a cascade of events. Such systems have been built to perform autonomous movements, e.g. the DNA walkers [2], rotary DNA devices [3], and DNA oscillators [4], and to act as fuel sources for non-covalent DNA catalysis reactions, as seen in some DNA amplification [5] and nucleated dendritic growth reactions [2]. Further, strand displacement reactions can be built to be responsive to pH [6] and to the presence of specific nucleic acid sequences [7]. However, it is not currently possible to design a priori nucleic acids that are responsive to small molecules. Instead, aptamer domains that have been selected from combinatorial libraries are required to provide sensitivity to specific small molecules. Additionally, the sensing conferred by the aptamer domain needs to be transduced to an output domain, a functionality that cannot be directly selected for with current technologies.

The reason why fusing aptamer and output domains together can work is because aptamers typically experience structural stabilization upon ligand binding often times leading to different base-pairing patterns. This simple effect can be exploited for engineering purposes. For example, ribozyme activity can be regulated by an incorporated aptamer sequence [8], strand displacement can be triggered by small molecule binding to a tethered aptamer domain [9], box shaped origami structures can release their contents in response to ligand binding to fused aptamer sequences [10], and gene expression can similarly be controlled through small molecule binding directly to the mRNA, as in the case of riboswitches. However, the merger of selected aptamers with designed strand displacement sequences has been difficult. Rather than selecting for aptamers that simply bind a specific ligand, a methodology that selects for sequences that undergo the desired structural change upon ligand binding would likely increase the success rate of developing small molecule triggered strand displacement systems.

2. A strand displacement based methodology

Recently, we developed an *in vitro* selection method to identify RNA molecules that interact with specific DNA duplex sequences in a ligand dependent manner [11]. The library is based on a previously selected thiamine pyrophosphate (TPP) responsive riboswitch variant +ThiM#2 [12]. Positions known to inactivate riboswitch activity are randomized. The selection is based on the liberation of the ribosome binding site (RBS) of the RNA upon ligand binding (Fig. 1). In the +ThiM#2 riboswitch, TPP binding causes a conformational change that allows the RBS sequence to become accessible. An accessible RBS is then capable of interacting with the toehold region of duplex DNA conjugated to magnetic beads, thereby resulting in both strand displacement and immobilization. Iterative rounds of selection allow for the isolation of RNA molecules that regulate RBS accessibility in response to ligand binding. In the protocol described herein, the RBS is inaccessible in the absence of the ligand and accessible in the presence of the ligand. In our proof-of-concept selection, the selected sequences are active in strand displacement and riboswitch activity.

There are several advantages to the described strand displacement based method. Rather than selecting for just binding, molecules that undergo the desired conformational change are directly selected. Although here the library is based on a pre-existing riboswitch sequence, it should be possible to implement this strategy with a fully randomized aptamer domain. In other words, rather than carrying out two separate selections, one for the aptamer domain and another for a transducer domain, as is the norm for the generation of synthetic riboswitches, only one selection would be needed. The protocol is carried out fully *in vitro*, making the method much faster to implement since *in vivo* screening steps are eliminated. The lack of *in vivo* steps also allows for the use of toxic ligands. Additionally, the sequence that base-pairs to the toehold can be easily changed to suit the specific needs of the desired system.

2.1. Assembling the DNA library

The DNA library is based on the ThiM#2 riboswitch [12]. The sequence is chosen because this riboswitch was previously thoroughly characterized [12]. Analogous efforts could use a library not based on a known riboswitch sequence, although this has not been tried yet. The library sequence should contain a transcriptional promoter. If the desired outcome is a riboswitch, then a RBS should be additionally incorporated. Downstream of the RBS element, an initiator strand (*I*) is introduced. This is the region that will mediate the strand displacement reaction and thus must be designed with the reporter sequence in mind. Here, the strand is 26 nucleotides long, avoiding a high GC content that could interfere with strand displacement (Table 1).

2.1.1.

The DNA library is assembled by PCR of overlapping oligonucleotides. The length of the oligonucleotides should not exceed 100 bp. The template sequence can be included in a plasmid and modified by introducing randomized positions. For example, here the +ThiM#2 riboswitch sequence is modified by PCR using a reverse primer containing four randomized positions. The forward primer does not carry any modification of the original sequence. 50 ng of template are amplified in a 50 μL reaction with 0.8 μM each oligonucleotide and 1.25 U AccuPrime Pfx DNA polymerase (Life Technologies). The cycling protocol follows the manufacturer's instructions for a three-step protocol with a few minor modifications. The initial denaturation step is at 95 °C for 30 s. Annealing is at 55 °C for 30 s, and the extension step is at 68 °C for 1 min/kb of the construct. The amplification is carried out for 25 cycles. Sterile water is preferable to diethyl pyrocarbonate (DEPC)-treated water for the PCR.

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