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# *In vitro* selection of antibiotic-binding aptamers

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ABSTRACT

Despite its wide applicability the selection of small molecule-binding RNA aptamers with high affinity binding and specificity is still challenging. We will present here a protocol which allows the *in vitro* selection of antibiotic-binding aptamers which turned out to be important building blocks for the design process of synthetic riboswitches. The presented methods will be compared with alternative *in vitro* selection protocols. A detailed note section will point out useful tips and pitfalls.



**Contents**



## 1. Introduction

Aptamers are short DNA- or RNA-oligonucleotides which are able to bind to many different molecules, such as small molecules, proteins, viruses and even entire cells, with high specificity and affinity. The complex three-dimensional structures they can fold into allow aptamers to form binding pockets and clefts like their protein counterparts [1,2]. Binding of the aptamer to its target results from structural compatibility, generated by stacking of aromatic rings, electrostatic and van der Waals contacts, and hydrogen bonding, or any combination of these interactions [3]. Many selected aptamers show affinities comparable to those observed for monoclonal antibodies. In addition, aptamers are able to recognize a distinct epitope on a target molecule and they can also discriminate between chiral molecules [4,5]. Thus, the differentiation between closely related target molecules (e.g. theophylline and caffeine) is possible [6]. Another advantage of aptamer generation is their "selectability" for binding to ligands beyond the spectrum of known natural systems by use of chemically

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produced oligonucleotide libraries, without the constraints imposed by a living organism. Furthermore, chemical synthesis allows the use of unusual or non-natural nucleotides, which even increases the possible complexity of aptamers [7,8].

In contrast to proteins, aptamers can be generated easily by *in vitro* selection or SELEX (Systematic Evolution of Ligands by EXponential Enrichment). Compared to antibodies, aptamers possess several advantages for their applicability based on the following features: (i) they can be selected by *in vitro* methods for their target molecules [9], (ii) aptamers can be chemically synthesized and modified using well-established nucleic acid chemistries [10], (iii) they are highly stable under elevated temperatures and, in addition, aptamers can be renatured after denaturation [11], (iv) they can be used as biosensors in combination with appropriate detection systems like electrochemical methods, surface plasmon resonance (SPR) or strand displacement [12] and (v) aptamers exhibit no toxicity and low immunogenicity [13]. Certainly, antibodies are well studied and have beside their nuclease resistance good pharmacokinetics and so antibodies are eligible for some applications [14].

SELEX was first described independently in 1990 at about the same time by three different laboratories. It is an iterative process of partitioning and amplification to extract aptamers from a large combinatorial library of randomized nucleic acid molecules. Ellington and Szostak isolated aptamers from a population of random RNA molecules that bind specifically to dyes that appear to mimic metabolic cofactors [9]. In the same year, Tuerk and Gold selected for RNA ligands that bind to bacteriophage T4 DNA polymerase [15] and Robertson and Joyce selected for an RNA enzyme, based on the *Tetrahymena* self-splicing group I intron, that specifically cleaves a single-stranded DNA substrate [16]. Focussing on RNA aptamers, they rely on simple building blocks like hairpins, bulges, internal loops and junctions to assume complex shapes, such as pseudoknots and quadruplexes, not the least because of RNAs exceptional properties to form unusual base parings and other specific interactions (for more information see [17]).

*In vitro* selection experiments start from an initial chemically synthesized and heavily amplified combinatorial library of DNA oligonucleotides, which has been transcribed into RNA. Usually, $10^{15}$ molecules are used to start such an experiment, thus giving the possibility to cover a large set of three-dimensional structures as well as target binding pockets. The core of the experimental procedure is the iterative incubation of this RNA pool with the target molecule and the following partitioning in binding and non-binding species. For this partitioning step, there are plenty of different possibilities established including filter assay, affinity chromatography, (capillary) electrophoresis or microfluidics [18–21].

Small molecule-binding aptamers can be exploited as biosensors, as recognition modules in riboswitches or even as antidotes in drug usage. Small molecules, such as toxins, carcinogens, pesticides, signalling molecules or antibiotics, are attractive but challenging target molecules for aptamer selection. They are well understood and most often pharmacologically characterized; they posses, at least in the case of antibiotics, low cytotoxicity, high solubility and are able to cross the cell membrane [22,23]. Small molecule-binding aptamers can be converted into biosensors or recognition modules in riboswitches. The growing need for the detection of traces of contaminations in food and feed, with e.g. antibiotics or toxins, and the necessity in synthetic biology to determine intracellular metabolite concentration for improving of metabolic pathways or genetic circuits by using small molecule-based biosensors requires the development of more and better small molecule-binding aptamers.

## 2. Method overview

Fig. 1 presents a general overview of the process used for *in vitro* selection of RNA aptamers. After design and synthesis of the template DNA pool, including a randomized region and a T7 promoter, the initial RNA library is generated by transcription using T7 RNA polymerase. In the SELEX process, this RNA library is subjected to iterative rounds of incubation with the immobilized ligand (not depicted), selecting for binding molecules, amplification via RT-PCR and conditioning of a new RNA library. After several rounds of selection, the RNA library shows a specific affinity for the ligand. At this point, the so called enriched library can be subsequently evaluated by cloning, sequencing and single clone analysis.

## 3. Material and methods

### 3.1. Large scale pool amplification

In order to transcribe the initial RNA pool for *in vitro* selection, it is necessary to generate the starting DNA pool from a chemically synthesized random DNA oligonucleotide library. For general considerations, see Section 4.1. Before starting the large scale PCR, optimization of the reaction conditions is recommended. The optimization of the PCR protocol is necessary to enhance yield and to avoid bias of the pool prior to selection (note 1). All improvement steps should aim for ideal amplification efficiency, so that in every PCR cycle the DNA amount is doubled. To validate the efficiency of a given PCR reaction, we recommend the use of qPCR (note 2). After PCR optimization, all components are mixed together as listed in Table 1 and aliquoted á 100 µl into standard 96-well PCR plates.

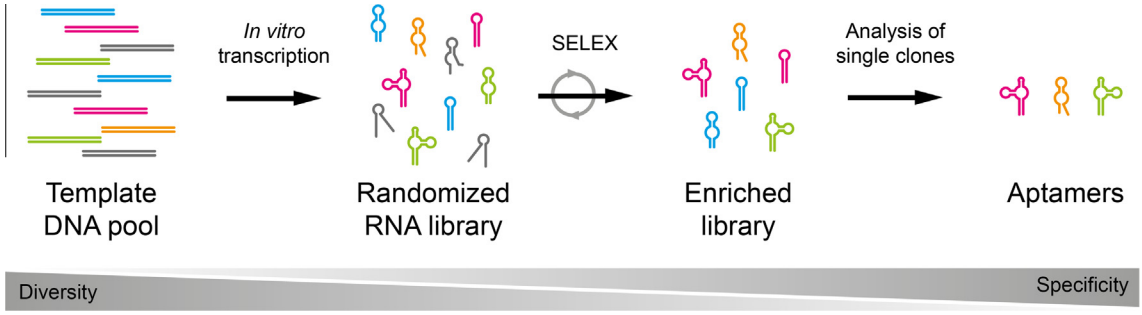


**Fig. 1.** Schematic overview of the SELEX process generating high affinity RNA aptamers. The template DNA pool is *in vitro* transcribed by T7 RNA polymerase into the naïve randomized RNA library. After iterative rounds of incubation of the RNA library with the immobilized ligand (not shown for clarity), partitioning in binding and non binding RNA molecules, RT-PCR and *in vitro* transcription, an enriched library is formed. This library can be evaluated through cloning, sequencing and single clone analysis. In the end, a few RNA molecules will show high affinity and specificity towards the ligand of choice.

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