Methods 106 (2016) 42–50

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

In vitro selection of antibiotic-binding aptamers

Florian Groher, Beatrix Suess *

Department of Biology, Technical University Darmstadt, Schnittspahnstr. 10, 64287 Darmstadt, Germany

ARTICLE INFO

Article history:
Received 16 February 2016
Received in revised form 10 May 2016
Accepted 15 May 2016
Available online 17 May 2016

Keywords:
In vitro selection
SELEX
RNA aptamer
Combinatorial chemistry
Biotechnology

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

* Corresponding author.
E-mail address: bsuess@bio.tu-darmstadt.de (B. Suess).

http://dx.doi.org/10.1016/j.ymeth.2016.05.008
1046-2023/

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.

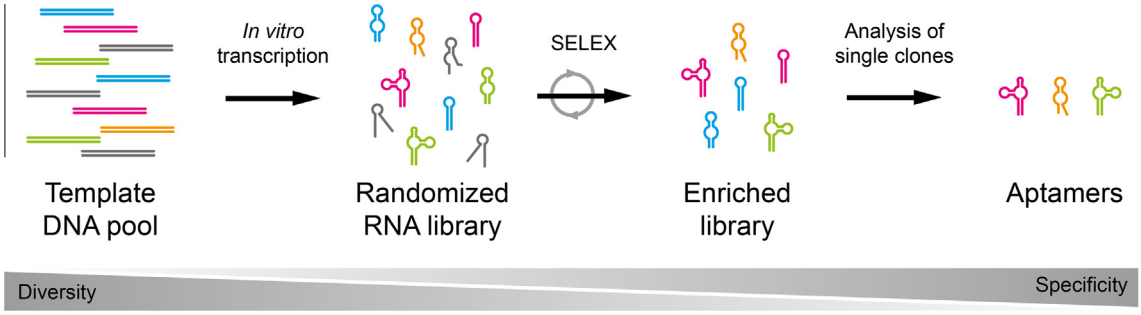

Fig. 1. Schematic overview of the SELEX process generating high affinity RNA aptamers. The template DNA pool is *in vitro* transcribed by T7 RNA polymerase into the naïve randomized RNA library. After iterative rounds of incubation of the RNA library with the immobilized ligand (not shown for clarity), 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.

Table 1
Reaction mixture for large scale pool amplification.

Component	Concentration
PCR buffer[#]	1×
MgCl2	1.5 mM
dNTPs (each)	0.2 mM
Pool_template	20 nM
Pool_fwd	5 μM
Pool_rev	5 μM
Taq DNA polymerase	50 U/mL

The oligonucleotides Pool_template, Pool_fwd and Pool_rev are discussed in Section 4.1. As noted in the text, the PCR conditions have to be optimized before starting with large scale pool amplification. This table summarizes standard reaction conditions that worked in most *in vitro* selections.

[#] 10× PCR buffer (100 mM Tris-Cl pH 9.0, 500 mM KCl, 1% Triton X-100).

Table 2
Reaction mixture for *in vitro* transcription.

Component	Concentration
Tris-Cl pH 8.0	40 mM
DTT	5 mM
$MgCl_2$	15 mM
ATP	2.5 mM
GTP	2.5 mM
CTP	2.5 mM
UTP	2.5 mM
DNA template	5 pool equivalents
T7 RNA polymerase	250 U/mL

If uncertain about the state of RNase contamination, the addition of RNase inhibitor to the reaction mixture can be beneficial. Beyond that, it has been reported that the addition of DTT to the reaction mixture reduces RNase activity [53].

The minimal required volume for this PCR is determined by the degree of randomization. For small pools, it is possible to cover the complete sequence space (e.g. randomized regions of <30 nucleotides), but for pools larger than 50 nt it becomes impossible to include all sequences in a single experiment. As mentioned above, it is not useful to exceed the amount of 1×10^{15} molecules per selection experiment due to unwanted RNA-RNA interactions at high RNA concentrations. However, in order to not bias the pool and to cover a certain sequence space statistically at the same time, it is important to start the PCR amplification with more than 1×10^{15} molecules to adequately sample the potential sequence space. This means that at least 5×10^{15} molecules (8.3×10^{-9} mol) [24] should be amplified, which leads, based on a template concentration of 200 nM (see Table 1), to an final PCR volume of 41.5 mL (8.3×10^{-9} mol ÷ 200×10^{-9} M = 0.0415 L).

Subsequently, the PCR reaction is concentrated by ethanol precipitation (note 3) and further purified by phenol/chloroform extraction (note 4) to remove traces of RNases.

3.2. In vitro transcription

For *in vitro* transcription of the initial RNA pool, all components are mixed together as listed in Table 2. The reaction is mixed by carefully pipetting up and down and is then incubated at 37 °C for 16 h (note 5). It is recommended to transcribe the initial RNA pool in a bigger batch for storage after purification in small aliquots suited for one selection experiment each. The minimal reaction volume should cover at least 5 times the quantity that it is used for one experiment, so that the pool's coverage is not biased.

3.3. RNA purification

The purification of the transcribed RNA pool is necessary to remove both template DNA and RNA species that are not full-length. The *in vitro* transcribed RNA is separated by polyacrylamide (PAA) gel electrophoresis. Hence, the RNA is dissolved in deionized formamide containing 25 mM EDTA (pH 8.0) before it is loaded onto a polyacrylamide gel containing 8 M urea (note 6). The buffer system for the gel is TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA). Following the gel run, the RNA is detected by UV shadowing. The full-length RNA is cut out, sliced into small pieces (about 0.2 cm × 0.2 cm) and eluted over night in 300 mM sodium acetate (pH 6.5) at 4 °C in an tube rotator. In order to remove PAA carryovers, the RNA is filtered through a 0.45 μM pore size filter and subsequently filled up with ethanol to an final concentration of 70% ethanol. The sample is incubated for 30 min at −20 °C, centrifuged (1 h, 17,000*g*, 4 °C) and washed twice (note 7). After air drying of the pellet (note 8), the RNA is dissolved in a suitable amount of RNase-free water (note 9).

After purification, the RNA has to be quality checked by gel electrophoresis. Finally, the randomization should be verified either by RNA-seq or RT-PCR with following subcloning and sequencing.

3.4. Immobilization of the ligand

The immobilization step summarized in this chapter is based on the publications describing the selection of neomycin and tetracycline aptamers [25,26]. The matrix, which was used for affinity chromatography, was epoxy-activated sepharose. This is a convenient way to immobilize ligands, because the epoxide group can easily react with molecules containing nucleophiles, including amines, sulfhydryls and hydroxyls (see Fig. 2). All known antibiotics contain at least one of these groups [27]. The only drawback of this method is the requirement for basic reaction conditions, which can be as high as pH 13. Not all ligands withstand these harsh conditions, so that other coupling methods must be taken into consideration (note 10 and Table 5).

The protocol that was used to immobilize neomycin and tetracycline is as follows. Five grams of epoxy-activated sepharose 6B (GE Healthcare) are hydrated according to the manufacturer's instructions. After that, the sepharose is incubated with 2 mM ligand in 10 mM NaOH (pH 11.5–12.0), in a total volume of 50 mL at 28 °C over night. The sepharose is then washed with alternate 60 mL volumes of 0.1 M NaOAc pH 4.0 and 0.1 M Tris-HCl pH 8.0 three times each and finally resuspended in 50 mL of 10 mM Tris-Cl pH 8.0 (notes 11 and 12). It can be stored at 4 °C for several weeks.

3.5. In vitro selection

As mentioned above, there are a plethora of different selection procedures, but the one that is widely used for small molecule selection which deliver the most reproducible results are based on affinity chromatography (see Section 4.2. passage affinity chromatography). Therefore, this section is derived to a great extent from the neomycin and tetracycline SELEX obtained with minor changes and some notes are given [25,26].

Prior to the selection experiment, a portion of the initial RNA pool needs to be radio labelled to monitor the overall process (note 13). For this purpose, the RNA is first dephosphorylated with antarctic phosphatase (NEB) (Table 3) and, following ethanol precipitation, radioactively phosphorylated with T4 polynucleotide kinase (NEB) and ^{32}P-γ-ATP (Table 4).

The 5′ radioactively end-labelled RNA (~220,000 cpm) and 5 μg unlabelled RNA are mixed in a total volume of 500 μL RNase-free water, denatured at 90 °C for 3 min and renatured at RT for 5 min (note 14). Following this, the RNA is diluted in 1 mL equilibration buffer (final concentrations: 10 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 250 mM NaCl). After washing a 1 mL bed volume of ligand-derivatized matrix with 10 vol of 1× equilibration buffer,

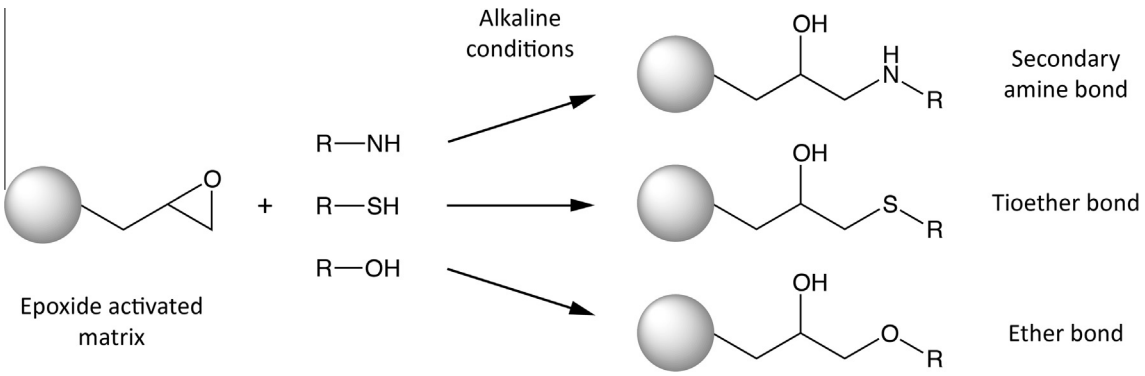

Fig. 2. Epoxide groups are reactive toward amines, sulfhydryls and hydroxyls. Under alkaline conditions amine-, sulfhydryl- and hydroxyl-containing ligands (abbreviated as R) can react with the epoxide activated matrix (grey-shaded ball) to secondary amines, thioether or ether bonds (adapted from Ref. [10]).

Table 3
Reaction mixture for RNA dephosphorylation.

Component	Concentration
Reaction buffer	1×
RNA	0.2 nmol
Antarctic phosphatase	0.1 U/μL

4 h @ 37 °C, 5 min @ 65 °C, ethanol precipitation, concentration measurement and quality check by gel electrophoresis.

Table 4
Reaction mixture for RNA phosphorylation.

Component	Concentration
Reaction buffer	1×
Dephosphorylated RNA	0.2 nmol
^{32}P-γ-ATP	15 nM
T4 polynucleotide kinase	0.2 U/μL

1 h @ 37 °C, ethanol precipitation, liquid scintillation counting and quality check by gel electrophoresis.

Table 5
Standard chemistries to immobilize ligands.

Functional group	Chemistry
Primary amino groups*	NHS esters Aldehydes Carbodiimides Epoxides Click§†
Sulfhydryl groups	Maleimides Haloacetyl derivatives‡ Epoxides
Carboxyl groups	Carbodiimides
Hydroxyl groups	Carbonyldiimidazole Isocyanates Epoxides

Summarized from [10,54].
* In some cases, secondary amino groups can also be coupled with the listed chemistries.
§ Phenylazides and perfluorinated phenylazides.
† Click chemistry can not only be applied to primary amino groups, but also to all other listed ones.
‡ Specifically iodoacetyls.

the sample is applied to the column and washed with 5 column volumes of equilibration buffer. The specifically bound RNA is eluted step-wise with 3 column volumes each of 1, 10, 100 mM ligand successively (for more details concerning this step see Section 4.2). One millilitre elution volumes are collected and quantified in a scintillation counter. The so eluted RNA is concentrated by ethanol precipitation (note 15), reverse-transcribed using a reverse transcriptase (e.g. M-MuLV, according to the manufacturers instructions) and amplified via PCR (same protocol as for large scale pool amplification, see Section 3.1). Transcription with T7 RNA polymerase (see Section 3.2) results in the RNA pool for the next selection round (see Fig. 3). The transcription steps from round 2 on are carried out in the presence of ^{32}P-α-UTP (body labelling) (note 16) to more easily follow the enrichment of the pool (see Fig. 3).

3.6. Cloning and single clone analysis

At the end of the *in vitro* selection, there is *hopefully* an enriched RNA pool that shows affinity and specificity for the ligand of choice. After the last round, the RNA is reverse-transcribed, amplified and subsequently cloned into an appropriate sequencing vector. We recommend using TOPO® TA cloning (ThermoFisher) to clone PCR products containing A-overhangs (note 17) at their 3′ termini for high efficiency subcloning. Following the subcloning, single bacterial colonies can be picked and prepared for sequencing. At least 50 colonies should be sequenced to get an idea of the distribution of sequences within the pool. In terms of the dropped sequencing cost of Next-Generation Sequencing technologies, it is worth considering the massive parallel sequencing of each selection round. After sequencing, clustering into families of different possible groups of aptamers can be done by hand or by using phylogenetic software (e.g. phylogeny.fr [28]). Binding motifs and the occurrence of these within the different sequences or families can be found with the MEME suite (http://meme-suite.org, [29]).

As a result of subcloning the RT-PCR products of the last round of selection, every aptamer within the sequencing vector contains the T7 promoter, so that run-off transcription from a linearized vector is easily possible [30]. The so transcribed RNA molecules can then be analysed for their binding properties. One possible method is to transcribe them and incorporate a radioactive label and then to apply them to the same columns used for *in vitro* selection. The chromatogram gives a rough idea of their ligand affinity (note 18). Besides affinity chromatography, several additional methods are available to rapidly screen these first possible aptamers: (i) fluorescence polarisation, (ii) SYBR green assay (change of fluorescence upon ligand binding), (iii) gold nanoparticle-based colorimetric assays (AuNP assay) or (iv) SPR. More about the workflow for aptamer screening can be found at McKeague et al. [31].

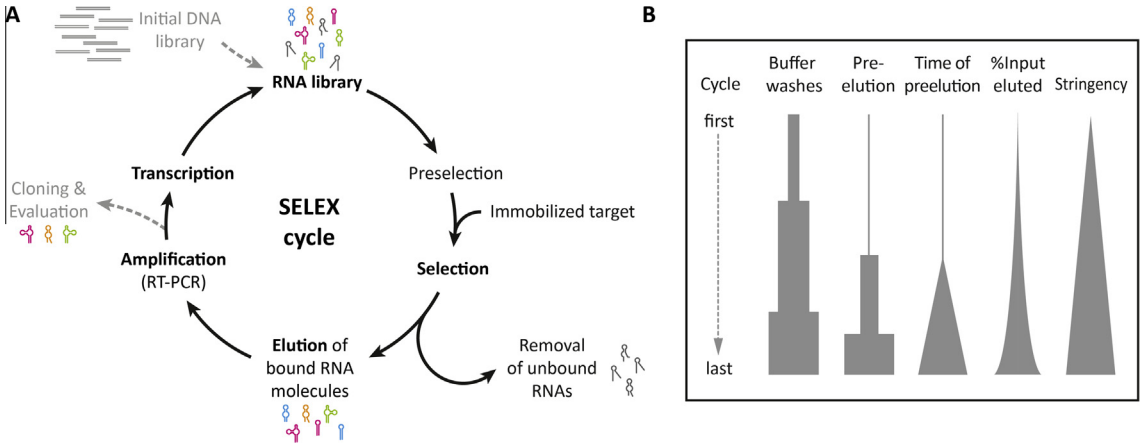

Fig. 3. Schematic overview of an *in vitro* selection experiment. (A) Beginning with the transcription of the naïve RNA pool from the initial DNA library, the RNA is incubated with a non-derivatized column (preselection). RNA, that does not interact with the column material, is incubated with the immobilized ligand (selection step). After incubation, non-bound molecules are removed and bound species are eluted from the column. The RNA pool for the next round of selection is generated by RT-PCR and transcription. After several rounds of selection, the RNA library shows an affinity to the immobilized ligand and can be evaluated (subcloning of the reverse transcribed pool, sequencing and single clone analysis; see Section 3.6). (B) General selection protocol. A general overview of the progressive stringency is shown in a schematic presentation. Starting from the first to the last round of selection, the number of buffer washes, pre-elution steps and time is increased as well as the amount of RNA that can be eluted. All of these parameters contribute to what is called selection stringency.

4. Theoretical considerations

4.1. Pool design

Usually, nucleic acid pools used for *in vitro* selection consist of a randomized core region flanked by constant sequences for PCR amplification, restriction digestion and *in vitro* transcription. Crucial for success in selection against small molecules is the length of the randomized region and the degree of randomization (note 19). Typically, about 5×10^{14}–1×10^{15} molecules are subjected to an *in vitro* selection experiment (note 20), which theoretically can cover all possible 25 mers ($4^{25} = 1.1 \times 10^{15}$). So, in principle it is not necessary to use pools with randomized regions larger than 25 nt to cover the complete possible sequence space. And indeed, no correlation between pool size and selection output was found, if randomized regions from roughly 20–140 nt were compared over all RNA aptamers published in Aptamer Base [32]. But there are some indications why larger randomized regions are of advantage: (i) pools larger than 25 nt give the possibility for complex three dimensional folding (not every nucleotide is directly involved in the binding event) and the option to form binding pockets for the ligand [33,34]; (ii) the most widely used RNA aptamers with dissociation constants in the lower nanomolar range and successful usage *in vivo* as so called riboswitches are aptamers that were selected from pools greater or equal than 40 nt of random sequence [6,25,26] and (iii) comparing synthetic aptamers with natural aptamer domains from riboswitches gives the hint that, despite loosing sequence information/coverage of the pool during selection, larger aptamer domains are more suitable for smaller ligands [35].

The constant regions are as important as the length of the randomized region. These regions facilitate the reverse transcription step, the following amplification step and *in vitro* transcription by introducing the T7 polymerase promoter (note 21). During the design process the following considerations should be taken into account: (i) length and melting temperature should be convenient for PCR amplification (note 22); (ii) PCR-associated artefacts should be avoided by exclusion of secondary-structure formation (hairpins) and self-association of the primers itself or with each other; (iii) for later analysis of the pool or for single clone analysis, restriction sites can be included to clone the enriched pool with or without constant regions into an appropriate sequencing vector. Beside these pivotal deliberations, there are several additional points on how to design good primers, that can be found in Sambrook et al. [36]. Assistance in primer design can be given by using Primer3 [37]. A typical example for a pool used for *in vitro* selection is shown in Fig. 4.

4.2. In vitro selection

Without any doubt, the most significant step in this method is the *in vitro* selection itself. There are many parameters which have to be considered before starting the selection process. One point, as already discussed, is the length of the randomized region and the nucleotide composition of the constant regions, but the buffer system, the concentration of magnesium ions, the matrix which is used for ligand immobilization, additives and the stringency of the selection process have also to be taken into account.

The buffer system is mostly based on user preference, but one would recommend to choose the buffer system with respect to

Fig. 4. Typical pool design for *in vitro* selection experiments. The pool template sequence is shown in black, the primers used for amplification of the initial pool and for amplification during SELEX are shown in red (referred to as Pool_fwd and Pool_rev), and the T7 polymerase promoter is shown in green. Restriction sites and the corresponding enzymes are highlighted in grey and noted. The randomized region is depicted in bold and shortened to N74 (adapted from Ref. [26]).

the final application the aptamers will be selected for. Deng et al. investigated the dependency of buffer composition, ionic strength and the influence of pH using the adenosine aptamer [38]. Comparing Tris-buffered systems with phosphate-buffered ones revealed no difference in binding or retention of the ligand on the column. Similarly, pH changes from 2.5 to 7.5 showed no significant effect on binding, but ionic strength and magnesium concentration did. It is obvious that the magnesium concentration should have a strong influence on the output of an selection experiment. On the one hand, Mg^{2+}-ions enable closer packing of the RNA by neutralising the negative charge of the backbone. This way, both tertiary structure formation and long-range interactions are more likely and, on the other hand, some ligands are bound in complex with a magnesium ion (e.g. tetracycline) [39]. The concentration of divalent cations, especially magnesium, in selection buffers was investigated by Carothers et al. in 2010 [40]. Here, selection experiments with two different ligands (p-amino phenylalanine and tetramethylrhodamine) were carried out at different magnesium concentrations, ranging from 1 mM to 10 mM. The aptamers, which were selected and investigated, showed better binding properties in 5 mM than in 1 mM Mg^{2+}. Additionally, titration experiments with the aptamers pAF-R1-1 and pAF-4Z1d3 demonstrated that not every aptamer needs high magnesium concentrations for functionality; but if it does, than 5 mM Mg^{2+} is sufficient to permit tight binding. Indeed, most selection experiments looking for aptamers that bind small molecules were carried out in the presence of 5 mM Mg^{2+} [41]. Coming back to the buffer composition, based on our experience we would recommend to use a Tris-buffered system with 5 mM $MgCl_2$ (10 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 250 mM NaCl).

The matrix used for ligand immobilization directly affects the overall selection process. In the last years, more and more modifications to the original SELEX procedure were established, such as Counter-SELEX, Deconvolution-SELEX, Photo-SELEX, Genomic-SELEX, EMSA-SELEX, CE- or microfluidic-SELEX [42]. They are all based on different immobilization matrices like agarose, sepharose, polyacrylamide or magnetic beads. Besides the matrix that is ultimately chosen for selection, there are some standard chemistries to covalently link the ligand of interest. A short overview over the different chemistries is given in Table 5.

As shown in Table 5, immobilization based on epoxides is one convenient way which can be chosen (note 23). As discussed above, the only drawback using epoxy-activated matrices are the alkaline conditions that are needed for the reaction, because not every ligand can withstand these harsh conditions. But on the other site, immobilization of an antibiotic with several functional groups can led to a derivatized matrix on which the ligand is immobilized via different positions, increasing the chance that groups that are necessary for RNA binding are not involved in coupling. Therefore, we recommend using the way of immobilization that is specified in Section 3.4.

The bullet point "SELEX additives" refers to substances that are meant to facilitate selection by suppressing unwanted and unspecific interactions. For this purpose, yeast tRNA or other RNA species, that are not amplifiable in the RT-PCR step, can be used as well as bovine serum albumin to minimize unspecific RNA protein interactions, if using streptavidin as immobilization matrix [43]. Furthermore, the use of heparin has been described to reduce non-specific binding of nucleic acids to proteins (if using streptavidin as immobilization matrix) [44].

The last point to be discussed is the stringency of the selection, which coincides with the selection progress and success. But what is stringency and how should it be applied to the selection process? Stringency is, first of all, not a fixed parameter and, second, stringency should be adjusted from round to round. Stringency is mostly applied to the selection process by varying the number of washing steps [33]. Importantly, not only this has to be taken into account, but also (i) the amount of ligand that is exposed to the RNA, thus also the ratio between RNA and immobilized ligand, (ii) the amount of specific or unspecific competitors, (iii) the (divalent) cation concentration, (iv) the equilibration time for binding and dissociation and (v) the type of elution.

The higher the amount of ligand, that is used to bind to the RNA, the easier it will be to capture RNA molecules, certainly including weak or unspecific binding molecules, too. In practice, at the beginning of a SELEX often higher ligand to RNA ratios are applied, but these then gradually decrease as the number of cycles increases [45]. Thus, the competition between each single RNA molecule increases and the strongest binder can become established. For more details regarding the ratio, take the mathematical models of Irvine et al. into account [46].

Longer equilibrium times give stronger binding species a greater chance to bind to the target, since weaker binding species more quickly dissociate from the ligand. In general, though, species with nanomolar dissociation constants or lower can be readily selected by allowing the reaction to equilibrate for five minutes or more. Usually up to 30 min are chosen for the binding reaction in order to allow for slow folding or refolding steps in the presence of the target. And from this point of view, stronger binding species can be retrieved, if the first elution fraction is discarded in order to get better values for K_{off} and, concomitantly, also get better K_D values [18]. Of course this is just a simple illustration what can be done to improve binding properties, affinities are a bit more complicated than that.

The elution process is an important part of the stringency discussion. Here, it can be clearly be chosen between specific and unspecific elution. Unspecific elution relies on resolving the aptamer structure by thermal or by chemical denaturation with urea or ethylenediaminetetraacetic acid (EDTA). This might be suitable for the first round of selection, but, in the later rounds, the RNA should be eluted using specific elution. A specific elution has the great advantage, that RNA species, that can be eluted by addition of free ligand to the selection buffer, are able to bind the ligand for sure, which excludes aptamers binding to the matrix. Furthermore, during selection the concentration of the free ligand can be reduced over time, contributing to a higher stringency [5,25,47].

The right selection round to stop and to evaluate the RNA pool is reached when, after several rounds of increased stringency, the pool shows a significant enrichment for target binding. It is important to note, that it should not be taken for granted, that the best aptamers can and will be found in this round. The possibility that the best binding aptamer is underrepresented or lost upon application of even higher stringency is not uncommon [48]. Hence, it may be worthwhile to evaluate different rounds of the *in vitro* selection experiment. For this, it might be interesting to determine the pool's complexity by restriction fragment polymorphism or melting curve analysis for example [49].

5. Notes

Note 1 PCR efficiencies that do not range between 90% and 110% may skew the representation of the pool and the sequence space is reduced due to bad PCR conditions. To increase amplification efficiency, the following parameters can be altered: (i) both primer and dNTP concentration (usually the concentration of both is higher than normally used in standard amplifications. Especially the amount of primer is increased to avoid mispriming or selfpriming of the amplifying pool); (ii) the magnesium concentration can be altered from 1 to 5 mM; (iii) the annealing temperature and annealing time and (iv) one last possibility is the re-design of the primers and also the constant regions of the pool.

Note 2 Make a serial dilution of the pool template starting from 20 nM. The dilution series should cover at least 5 orders of magnitudes with three replicates. After the run, plot the amount of template against the corresponding C_q-value. By linear regression of the data points, the slope can be utilized to calculate the PCR efficiency by using the following formula.

$$E = 10^{-1/slope}$$

In an ideal situation, E equals 2, so that in every amplification cycle the number of DNA molecules gets doubled. In that case the slope of the standard curve will be −3.32 and the PCR efficiency is 100%. Amplification efficiency is also frequently presented as a percentage, that is, the percent of template that was amplified in each cycle. The percentage can be calculated with

$$\%E = (E - 1) \times 100\%$$

PCR efficiencies ranging from 90 to 110% are acceptable.

Note 3 One volume nucleic acid solution is mixed with 1/10 vol sodium acetate (pH 6.5) and 2.5 vol ethanol. After vortexing and incubation at −20 °C for 30 min, the precipitation mixture is centrifuged (1 h, 17,000*g*, 4 °C). Afterwards, the supernatant is removed and the pellet is washed with 70% (v/v) ethanol and centrifuged again. Once again, the supernatant is removed and the pellet is air-dried and dissolved in a suitable amount of RNase-free water.

Note 4 One volume of phenol:chloroform:isoamyl alcohol (25:24:1) is added to the precipitated PCR reaction, the sample is transferred into a MaXtract tube (Qiagen) and centrifuged. The aqueous phase is transferred into a new MaXtract tube, 1 vol of chloroform:isoamyl alcohol (24:1) is added and the sample is centrifuged again. The supernatant is transferred into a new reaction tube, the DNA is precipitated by adding 1 vol isopropyl alcohol and 0.1 vol of sodium acetate (3 M, pH 6.5). Afterwards it is incubated at −20 °C for 30 min and pelleted by centrifugation. The pellet is washed with 70% (v/v) ethanol, centrifuged and after drying the pellet at 37 °C it is resolved in a suitable amount of RNase-free water. Subsequently, the prepared DNA pool is controlled via denaturating PAGE.

Note 5 The success of the transcription reaction can sometimes be monitored by observing the formation of a white precipitate of magnesium pyrophosphate that is released from each polymerized ribonucleotide. However, not every successful transcription shows this precipitation. Additionally, supplementing inorganic pyrophosphatase prevents precipitate formation and greatly increases the yield of *in vitro* transcription reaction by shifting the reaction equilibrium towards polymerization product.

Note 6 Desalting the *in vitro* transcription mixture just before loading onto the denaturing PAGE will lead to sharper product bands and better resolution of the entire gel.

Note 7 The size of the polyacrylamide gel is based on the amount of RNA that has to be separated. Similarly, the percentage of the gel should be chosen on basis of pool length. For example, good results for 100 nt long RNAs are achieved with 6% PAA/8 M Urea in TBE (pH8.0).

Note 8 Sometimes the RNA is not completely pelleted, but a gel-like matrix is formed. This is the RNA, so do not discard. After addition of 70% (v/v) ethanol and vortexing, the RNA pellets completely during the following centrifugation step.

Note 9 If the RNA is dried for too long, it is not possible to resuspend it. For that reason it should not be dried at temperatures exceeding 37 °C.

Note 10 It is highly recommended to use RNase-free water. If it is necessary to use buffer, avoid divalent cations like magnesium or calcium. These ions might trap the RNA in unwanted conformations and the subsequent folding step prior to the selection experiment cannot be done without RNA degradation.

Note 11 After the coupling process and for calculation of the coupling efficiency, it is necessary to keep an aliquot from every wash fraction/supernatant and measure it under appropriate conditions in a spectrophotometer or fluorometer. If the ligand itself is fluorescent, then the amount of immobilized ligand can be directly quantified on the matrix by using fluorescence measurements.

Note 12 When using this protocol or an NHS-ester derivatized matrix, it can be useful to block all remaining activated groups by applying 1 M ethanolamine (pH 8.0) to the column material. Afterwards, the matrix again needs to be washed three times with alternating 0.1 M NaOAc pH 4.0 and 0.1 M Tris-Cl pH 8.0.

Note 13 Instead of body labelling other methods can be used to follow reaction process. For example, fluorescent labelles can be used or (q)PCR analysis after each round. The great advantage of labelling RNA with radioactive ^{32}P-α-UTP during standard transcription (body labelling) is the possibility to track the complete selection process, from column loading over washing to eluting bound aptamers. Same can be done with fluorescence labels, but this would need an additional enzymatic step. On the other hand, qPCR analysis needs a far greater technical effort and by doing on-step qPCR it is hard to cover the complete process in terms of cost.

Note 14 The fact that RNA molecules have an amazing flexibility with respect to structure as well as to function, can cause problems concerning their proper folding into a single native structure [50]. This dilemma is known as the RNA-folding problem [51]. RNA can easily misfold and become trapped in certain, sometimes unwanted, conformations that can be very stable and tenacious. To avoid this problem and to be sure that the RNA has the same conformation in every selection round or later on during single clone analysis, it is recommended to subject RNA to a well defined folding program. Usually the RNA is heated to 95 °C for 5 min in the absence of divalent cations and then cooled to 4 °C. After that, selection or so called equilibration buffer is added and the RNA is warmed to room temperature for several minutes.

Note 15 To facilitate precipitation of RNA, especially in the first rounds, in which the RNA concentrations are low, the addition of a carrier (e.g. yeast tRNA or glycogen) can be useful.

Note 16 For body labelling, ^{32}P-α-UTP is additionally added to the transcription reaction. Usually, the radioactively labelled UTP is mixed at 1/10 of the amount of the standard concentration of NTPs.

Note 17 A-overhangs on 3′ ends are generated by *Taq* DNA polymerase facilitating ligation into TOPO® TA cloning vectors. On the other hand, proofreading DNA polymerases with 3′ to 5′ exonuclease activity like Phusion or Q5 DNA polymerase, do not create this overhang. Prior to cloning this kind of PCR products into a TOPO® TA cloning vector, A-overhangs must be added to the PCR product.

Note 18 Affinity chromatography, as used in the *in vitro* selection, is a good approach to reduce the number of putative aptamers sequences to a minimum before utilizing more laborious and cost-intensive methods. Despite the fact, that this type of experiment does not allow binding constants or thermodynamic parameters to be extracted, it is still the best way to exclude the event of non-binding because the same procedure like in the generation of this aptamer is used.

Note 19 There are two possibilities on randomization of a SELEX pool: (i) complete randomization or (ii) partial randomization. The latter one can consists either of a randomized region interrupted by a defined sequence or every nucleotide position is partially mutated based on a given sequence (doped pool).

Note 20 It is indeed possible to easily synthesize more than 1×10^{15} molecules and transcribe them into an RNA pool, but using more RNA than 1×10^{15} molecules will lead to unwanted

unspecific RNA-RNA interactions (RNA concentrations in the mM range), if the reaction volume is not drastically increased. By doing this, selection procedure is not longer easy to handle.

Note 21 It was shown that transcription from the T7 minimal promoter gives good yields, but the addition of several upstream nucleotides facilitates transcription. Additionally, take note that the dinucleotide GG following the T7 promoter is necessary for proper transcription initiation and cannot be skipped. Every transcript has to start with at least one G [52].

Note 22 The length of a typical PCR primer is between 17 and 23 nt and the ideal melting temperature is around 58 °C. Higher annealing temperatures increases specificity of the PCR reaction and should be preferred.

Note 23 The possibility of utilization of the streptavidin-biotin interaction should be kept in mind, but the initial immobilization step, that is the biotinylation of the ligand, is based on the same standard chemistries.

Acknowledgments

The authors thank Christian Berens for critically reading the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SFB902 A2) and EU FP7-KBBE-2013-7 No. 613745, Promys and ERASynBio "Intensify".

References

[1] T. Hermann, D.J. Patel, RNA bulges as architectural and recognition motifs, Structure 8 (2000) R47–R54.
[2] D.J. Patel, A.K. Suri, F. Jiang, L. Jiang, P. Fan, R.A. Kumar, et al., Structure, recognition and adaptive binding in RNA aptamer complexes, J. Mol. Biol. 272 (1997) 645–664, http://dx.doi.org/10.1006/jmbi.1997.1281.
[3] T. Hermann, D.J. Patel, Adaptive recognition by nucleic acid aptamers, Science 287 (2000) 820–825.
[4] M. Michaud, E. Jourdan, A. Villet, A. Ravel, C. Grosset, E. Peyrin, A DNA aptamer as a new target-specific chiral selector for HPLC, J. Am. Chem. Soc. 125 (2003) 8672–8679, http://dx.doi.org/10.1021/ja034483t.
[5] A. Geiger, P. Burgstaller, H. von der Eltz, A. Roeder, M. Famulok, RNA aptamers that bind L-arginine with sub-micromolar dissociation constants and high enantioselectivity, Nucl. Acids Res. 24 (1996) 1029–1036.
[6] R.D. Jenison, S.C. Gill, A. Pardi, B. Polisky, High-resolution molecular discrimination by RNA, Science 263 (1994) 1425–1429.
[7] G.M. Stovall, R.S. Bedenbaugh, S. Singh, A.J. Meyer, P.J. Hatala, A.D. Ellington, et al., In vitro selection using modified or unnatural nucleotides, Curr. Protoc. Nucl. Acid Chem. 56 (2014) 9.6.1–9.6.33, http://dx.doi.org/10.1002/0471142700.nc0906s56.
[8] S.M. Knudsen, M.P. Robertson, A.D. Ellington, In vitro selection using modified or unnatural nucleotides, Curr. Protoc. Nucl. Acid Chem. (2002). Chapter 9 Unit 9.6. doi:10.1002/0471142700.nc0906s07.
[9] A.D. Ellington, J.W. Szostak, In vitro selection of RNA molecules that bind specific ligands, Nature 346 (1990) 818–822, http://dx.doi.org/10.1038/346818a0.
[10] G.T. Hermanson, Bioconjugate techniques, in: G.T. Hermanson (Ed.), Bioconjugate Techniques, second ed., Academic Press, New York, 2008, http://dx.doi.org/10.1016/B978-0-12-382239-0.00005-4. pp. v–.
[11] M. Mascini, Aptamers and their applications, Anal. Bioanal. Chem. 390 (2008) 987–988, http://dx.doi.org/10.1007/s00216-007-1769-y.
[12] M. McKeague, M.C. DeRosa, Challenges and opportunities for small molecule aptamer development, J. Nucl. Acids 2012 (2012) 1–20, http://dx.doi.org/10.1021/bi061613c.
[13] C.R. Ireson, L.R. Kelland, Discovery and development of anticancer aptamers, Mol. Cancer Ther. 5 (2006) 2957–2962, http://dx.doi.org/10.1158/1535-7163.MCT-06-0172.
[14] S. Dübel, Handbook of Therapeutic Antibodies, Wiley-VCH, 2007.
[15] C. Tuerk, L. Gold, Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, Science 249 (1990) 505–510.
[16] D.L. Robertson, G.F. Joyce, Selection in vitro of an RNA enzyme that specifically cleaves single-stranded DNA, Nature 344 (1990) 467–468, http://dx.doi.org/10.1038/344467a0.
[17] R. Batey, R. Rambo, J. Doudna, Tertiary motifs in RNA structure and folding, Angew. Chem. Int. Ed. Engl. 38 (1999) 2326–2343.
[18] B. Hall, S. Arshad, K. Seo, C. Bowman, M. Corley, S.D. Jhaveri, In Vitro Selection of RNA Aptamers to a Protein Target by Filter Immobilization, John Wiley & Sons Inc., Hoboken, NJ, USA, 2001, http://dx.doi.org/10.1002/0471142700.nc0903s00.
[19] M. McKeague, C.R. Bradley, A. De Girolamo, A. Visconti, J.D. Miller, M.C. DeRosa, Screening and initial binding assessment of fumonisin b(1) aptamers, Int. J. Mol. Sci. 11 (2010) 4864–4881, http://dx.doi.org/10.3390/ijms11124864.
[20] A.P. Drabovich, M. Berezovski, V. Okhonin, S.N. Krylov, Selection of smart aptamers by methods of kinetic capillary electrophoresis, Anal. Chem. 78 (2006) 3171–3178, http://dx.doi.org/10.1021/ac060144h.
[21] X. Lou, J. Qian, Y. Xiao, L. Viel, A.E. Gerdon, E.T. Lagally, et al., Micromagnetic selection of aptamers in microfluidic channels, Proc. Natl. Acad. Sci. USA 106 (2009) 2989–2994, http://dx.doi.org/10.1073/pnas.0813135106.
[22] A.J. Smith, New horizons in therapeutic antibody discovery: opportunities and challenges versus small-molecule therapeutics, J. Biomol. Screening 20 (2015) 437–453, http://dx.doi.org/10.1177/1087057114562544.
[23] D. Steinhilber, M. Schubert-Zsilavecz, H.J. Roth, Medizinische Chemie, Wissenschaftliche Verlagsgesellschaft Stuttgart, 2010.
[24] B. Hall, J.M. Micheletti, P. Satya, K. Ogle, J. Pollard, A.D. Ellington, Design, synthesis, and amplification of DNA pools for in vitro selection, Curr. Protoc. Nucl. Acid Chem. (2009), http://dx.doi.org/10.1002/0471142700.nc0902s39. Unit 9.2, Chapter 9.
[25] C. Berens, A. Thain, R. Schroeder, A tetracycline-binding RNA aptamer, Bioorg. Med. Chem. 9 (2001) 2549–2556.
[26] M.G. Wallis, U. von Ahsen, R. Schroeder, M. Famulok, A novel RNA motif for neomycin recognition, Chem. Biol. 2 (1995) 543–552.
[27] Sigma-Aldrich, Antibiotics A to Z, Sigmaaldrich.com. (n.d.). <http://www.sigmaaldrich.com/life-science/biochemicals/biochemical-products.html?TablePage=14572854> (accessed January 7, 2016).
[28] A. Dereeper, V. Guignon, G. Blanc, S. Audic, S. Buffet, F. Chevenet, et al., Phylogeny.fr: robust phylogenetic analysis for the non-specialist, Nucl. Acids Res. 36 (2008) W465–W469, http://dx.doi.org/10.1093/nar/gkn180.
[29] T.L. Bailey, M. Boden, F.A. Buske, M. Frith, C.E. Grant, L. Clementi, et al., MEME SUITE: tools for motif discovery and searching, Nucl. Acids Res. 37 (2009) W202–W208, http://dx.doi.org/10.1093/nar/gkp335.
[30] R.K. Hartmann, A. Bindereif, A. Schön, E. Westhof, Handbook of RNA Biochemistry, John Wiley & Sons, 2015.
[31] M. McKeague, A. De Girolamo, S. Valenzano, M. Pascale, A. Ruscito, R. Velu, et al., Comprehensive analytical comparison of strategies used for small molecule aptamer evaluation, Anal. Chem. 87 (2015) 8608–8612, http://dx.doi.org/10.1021/acs.analchem.5b02102.
[32] M. McKeague, E.M. McConnell, J. Cruz-Toledo, E.D. Bernard, A. Pach, E. Mastronardi, et al., Analysis of in vitro aptamer selection parameters, J. Mol. Evol. (2015), http://dx.doi.org/10.1007/s00239-015-9708-6.
[33] K.A. Marshall, A.D. Ellington, In vitro selection of RNA aptamers, Methods Enzymol. 318 (2000) 193–214.
[34] S. Klussmann, The Aptamer Handbook, Wiley-VCH, 2006.
[35] R.R. Breaker, Riboswitches and the RNA world, Cold Spring Harbor Perspect. Biol. 4 (2012), http://dx.doi.org/10.1101/cshperspect.a003566.
[36] J. Sambrook, D.W. Russell, Molecular Cloning, CSHL Press, 2001.
[37] A. Untergasser, I. Cutcutache, T. Koressaar, J. Ye, B.C. Faircloth, M. Remm, et al., Primer3 – new capabilities and interfaces, Nucl. Acids Res. 40 (2012) e115, http://dx.doi.org/10.1093/nar/gks596.
[38] Qing Deng, Igor German, A. Danielle Buchanan, R.T. Kennedy, Retention and separation of adenosine and analogues by affinity chromatography with an aptamer stationary phase, Anal. Chem. (2001).
[39] H. Xiao, T.E. Edwards, A.R. Ferré-D'Amaré, Structural basis for specific, high-affinity tetracycline binding by an in vitro evolved aptamer and artificial riboswitch, Chem. Biol. 15 (2008) 1125–1137, http://dx.doi.org/10.1016/j.chembiol.2008.09.004.
[40] J.M. Carothers, J.A. Goler, Y. Kapoor, L. Lara, J.D. Keasling, Selecting RNA aptamers for synthetic biology: investigating magnesium dependence and predicting binding affinity, Nucl. Acids Res. 38 (2010) 2736–2747, http://dx.doi.org/10.1093/nar/gkq082.
[41] J. Cruz-Toledo, M. McKeague, X. Zhang, A. Giamberardino, E. McConnell, T. Francis, Aptamer base: a collaborative knowledge base to describe aptamers and SELEX experiments, Database (Oxford) 2012 (2012) base006, http://dx.doi.org/10.1093/database/bas006.
[42] R. Stoltenburg, C. Reinemann, B. Strehlitz, SELEX—a (r)evolutionary method to generate high-affinity nucleic acid ligands, Biomol. Eng. 24 (2007) 381–403, http://dx.doi.org/10.1016/j.bioeng.2007.06.001.
[43] C.W.J. Smith, RNA-Protein Interactions, Oxford University Press, USA, 1998.
[44] K. Bendak, F.E. Loughlin, V. Cheung, M.R. O'Connell, M. Crossley, J.P. Mackay, A rapid method for assessing the RNA-binding potential of a protein, Nucl. Acids Res. 40 (2012) e105, http://dx.doi.org/10.1093/nar/gks285.
[45] A. Ozer, B.S. White, J.T. Lis, D. Shalloway, Density-dependent cooperative non-specific binding in solid-phase SELEX affinity selection, Nucl. Acids Res. 41 (2013) 7167–7175, http://dx.doi.org/10.1093/nar/gkt477.
[46] D. Irvine, C. Tuerk, L. Gold, SELEXION. Systematic evolution of ligands by exponential enrichment with integrated optimization by non-linear analysis, J. Mol. Biol. 222 (1991) 739–761.
[47] J.R. Lorsch, J.W. Szostak, In vitro selection of RNA aptamers specific for cyanocobalamin, Biochemistry 33 (1994) 973–982.
[48] T. Schütze, B. Wilhelm, N. Greiner, H. Braun, F. Peter, M. Mörl, et al., Probing the SELEX process with next-generation sequencing, PLoS One 6 (2011) e29604, http://dx.doi.org/10.1371/journal.pone.0029604.
[49] N. Mencin, T. Šmuc, M. Vraničar, J. Mavri, M. Hren, K. Galeša, et al., Optimization of SELEX: comparison of different methods for monitoring the progress of in vitro selection of aptamers, J. Pharm. Biomed. Anal. 91 (2014) 151–159, http://dx.doi.org/10.1016/j.jpba.2013.12.031.

[50] R. Schroeder, A. Barta, K. Semrad, Strategies for RNA folding and assembly, Nat. Rev. Mol. Cell Biol. 5 (2004) 908–919, http://dx.doi.org/10.1038/nrm1497.
[51] D. Herschlag, RNA chaperones and the RNA folding problem, J. Biol. Chem. 270 (1995) 20871–20874.
[52] J.F. Milligan, D.R. Groebe, G.W. Witherell, O.C. Uhlenbeck, Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates, Nucl. Acids Res. 15 (1987) 8783–8798.
[53] Z. Chen, J. Ling, D. Gallie, RNase activity requires formation of disulfide bonds and is regulated by the redox state, Plant Mol. Biol. 55 (2004) 83–96, http://dx.doi.org/10.1007/s11103-004-0438-1.
[54] G.T. Hermanson, A.K. Mallia, P.K. Smith, Immobilized affinity ligand, Techniques (1992).