Chapter 2

In Vitro Selection of RNA Aptamers for a Small-Molecule Dye

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Abstract

Artificial riboswitches can be generated by functional coupling of an RNA aptamer for synthetic small molecule to an expression platform. RNA aptamers that can bind strongly and selectively to their target are feasible to be used for obtaining more potent artificial riboswitches. In this chapter, we describe tips and notes for in vitro selection of RNA aptamers targeting synthetic small molecules.

Key words RNA aptamer, In vitro selection, Small molecule, Riboswitch, Chemical biology

1 Introduction

Bacteria and some eukaryotes use natural RNA aptamers for metabolite sensing and gene regulation [1–3]. These RNA aptamers specifically bind to their targets and exhibit conformational changes that are transduced into the changes in the secondary structure of a downstream expression platform, resulting in a transcriptional or a translational modulation of gene expression. This RNA-based gene regulatory system, termed riboswitch, was first experimentally validated by RR Breaker in 2002 [4]. To date, more than 20 classes of natural riboswitches have been identified in many species [3].

The discovery of natural riboswitches has encouraged greater interest among researchers who seek to create a new type of gene expression system. In fact, a number of studies have demonstrated the creation of "artificial riboswitches" for the conditional control of gene expression in bacteria by engineering natural riboswitches [5–7]. On the other hand, in vitro-selected RNA aptamers for synthetic small molecules have also been employed to create artificial riboswitches [8–12].

In general, the use of RNA aptamers selected against synthetic small molecules for controlling gene expression might offer several advantages: orthogonality to endogenous system and choice of a

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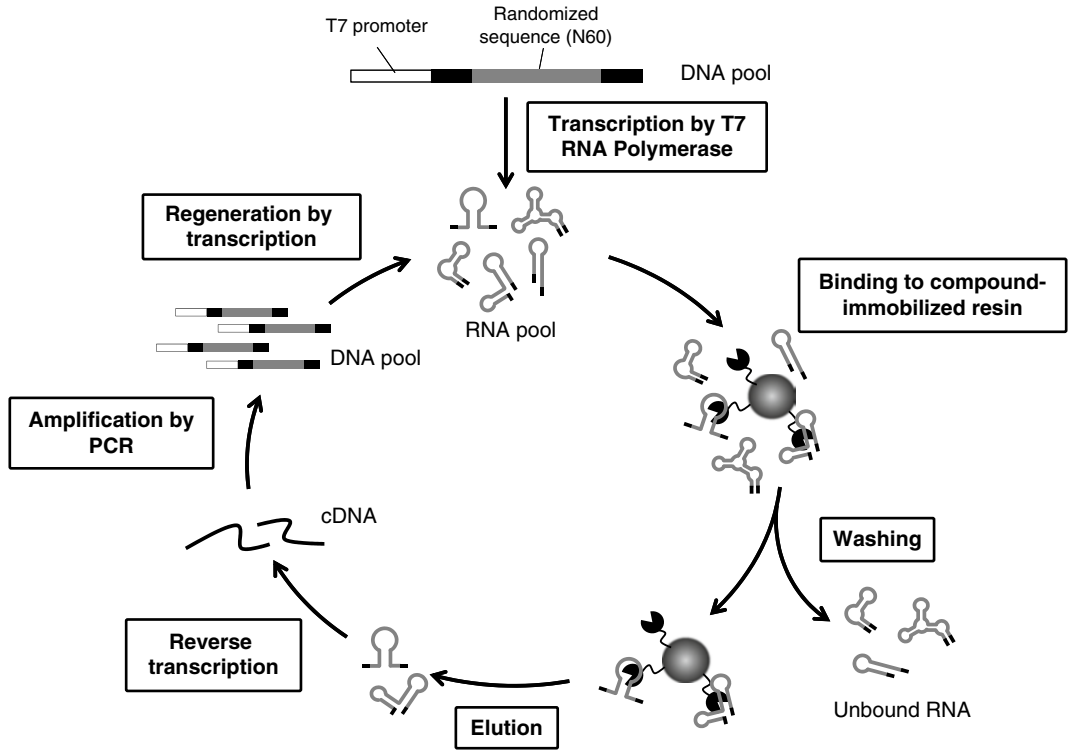

Fig. 1 General scheme of in vitro selection

wide range of target ligands. RNA aptamers can be isolated from a pool of random RNA sequences through a process called in vitro selection or Systematic Evolution of Ligands by Exponential enrichment (SELEX) (Fig. 1) [13]. In vitro selection experiments consist of several steps: (1) preparation of a ligand-immobilized matrix, (2) transcription of RNA from a synthetic pool of double-stranded DNA, (3) isolation of RNA species that can specifically bind to the ligand on the matrix, and (4) regeneration of dsDNA pools through reverse transcription and PCR amplification. Steps 2–4 must be repeated several times to yield aptamers having higher binding affinity to the ligand. Here we focus on steps 1 and 3 that can mostly affect the successful selection of high-affinity aptamers from a random RNA pool and describe notes and tips for these steps.

In the first step of an in vitro selection experiment, an affinity matrix is prepared by immobilizing a target ligand on a resin. Although there is no consensus for good targets for in vitro selection, several considerations may help researchers to choose target ligands. Ligands that contain chemical moieties that are complementary to those of nucleic acids should be good targets. For example, in vitro selections against aromatic compounds, heterocyclic compounds, or nucleotide analogs have often been

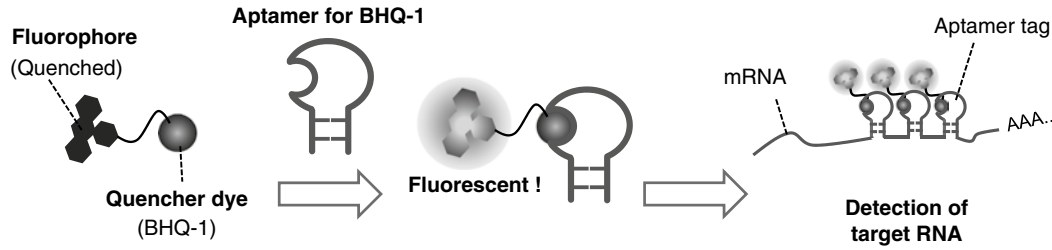

Fig. 2 Application of in vitro-selected aptamers against a small-molecule dye for RNA tagging

successful. In addition, positively charged functional groups (e.g., primary amino groups) of a ligand may facilitate an aptamer selection, as they increase binding affinity to RNA by electrostatic interaction with the phosphate backbone. Next, the ligand of choice can be attached to a resin for making a ligand-immobilized matrix. There are two major methods available for ligand attachment: one is covalent coupling of ligands to a reactive functional group on matrix and another is immobilization of biotinylated ligands on avidin-coated matrix. Typically, the former coupling method gives the matrix with higher concentration of ligand (~20 mM) than that of the latter one (~100 nM), although it may depend on the concentration of functional groups that were originally available and the coupling efficiency. The concentration of the ligand on the matrix can significantly influence the course of a selection experiment. Decreasing the concentration of the ligand on the matrix increases the chance for having aptamers that bind more strongly to the target ligand, encouraging competition for a smaller number of binding sites.

Binding and elution conditions also directly affect the stringency of the selections. Binding buffer containing monovalent and divalent cations (Na^+, K^+, and Mg^{2+}) may be used to stabilize RNA structures, which promotes the formation of specific aptamer structures or interactions with the ligand. Negative selection to remove matrix-binding species using an underivatized resin is critically important to obtain specific aptamers, because it may be difficult to select aptamers if once the matrix binders accumulate and become dominant. To obtain specific RNA aptamers to the target ligand, affinity elution with the specific ligand is highly preferred, though denaturing agents such as 6–8 M urea and 4 M guanidine can elute bound RNA from the matrix by denaturing secondary structures of RNA.

We present our recent work on in vitro selection of RNA aptamers targeting a small-molecule dye (Black hole quencher-1: BHQ-1). Our study has focused on utilizing the aptamers as a tag for RNA detection in living cells in combination with the designed fluorescent probe (Fig. 2) [14]. In vitro selection was performed to isolate aptamers for BHQ-1 from an RNA pool containing a

60-base random sequence. After 13 rounds of selection against a BHQ-1-immobilized resin, we obtained four RNA sequences (A1–A4) that shared a conserved sequence of 17 nucleotides. A1–A3 had similar K_d values around 5 μM, whereas A4 that contained a one-base mismatch in the conserved sequence had three times lower affinity than A1–A3, suggesting that the sequence of the conserved region is important in the recognition of BHQ-1. A1–A4 were predicted to form a stem–loop, with the conserved sequence in the loop region. The prevention of stem–loop formation caused a marked decrease in binding affinity of these aptamers, indicating that the stem–loop is the minimal binding motif. In fact, a short version of A1 containing only the stem–loop retained binding ability to BHQ-1 with lower affinity than parental A1. Using A1 sequence as a tag, a specific mRNA was successfully detected by restoration of fluorescence probe in an *E. coli* lysate.

2 Materials

2.1 Equipment

1. Thermal cycler.
2. Horizontal and vertical electrophoresis system.
3. UV transilluminator.
4. Tabletop centrifuge.
5. Real-Time PCR System (7500 Fast Real-Time PCR System, Applied Biosystems).
6. Microplate reader (MTP-800, CORONA).
7. Fluorophotometer (LS-55, PerkinElmer).

2.2 Reagents and Solutions

1. BHQ-1 carboxylic acid, BHQ-1 amine (Bioresearch Technologies).
2. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride: DMT-MM (KOKUSAN CHEMICAL Co., Ltd).
3. Triethylamine (Wako Pure Chemical Industries, Ltd).
4. Platinum® *Pfx* DNA polymerase (Invitrogen).
5. 10 mM dNTP mix (Invitrogen).
6. T7 RNA polymerase (TAKARA).
7. 25 mM Nucleotides rNTPs Mixture (TOYOBO).
8. RQ1 DNase I (Promega).
9. Prime Script reverse transcriptase (TAKARA).
10. DNA Ligation Kit (TAKARA).
11. Plasmid isolation kit (QIAGEN).
12. MEGAscript T7 Kit (Ambion).
13. In-Fusion Dry-Down PCR Cloning Kit (Clontech Laboratory Inc.).

14. RTS 100 *E. coli* HY Kit, 5 (Prime Inc.).
15. 3 M sodium acetate solution (pH 5.2).
16. 7.5 M ammonium acetate solution (Sigma-Aldrich).
17. Annealing buffer: 10 mM Tris–HCl, pH 7.5, 100 mM KCl, 50 mM NaCl.
18. Binding buffer: 10 mM Tris–HCl, pH 7.5, 100 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$.
19. 250 mM $MgCl_2$.

2.3 Supplies

1. Microtubes, 1.5 and 2.0 mL.
2. Falcon tubes, 15 and 50 mL.
3. 0.2 mL PCR tubes.
4. EAH sepharose™ 4B resins (GE Healthcare).
5. Poly-Prep Chromatography Columns (BIO-RAD).
6. Micro Bio-Spin Chromatography Columns (BIO-RAD).
7. NAP-5 columns (GE Healthcare).
8. 96-well glass bottom black plates (655896, Greiner Bio-One).

3 Methods

All solutions and buffers should be made with DEPC-treated water. Use RNase/DNase-free disposable plasticware and micropipet tips where possible.

3.1 Preparation of BHQ-1-Immobilized Resin

1. Place 0.5 mL of EAH sepharose™ 4B resin in a Poly-Prep Chromatography Column and allow to drain by gravity.
2. Wash the resin thoroughly with at least three bed volumes of dioxane–H_2O (3:1, v/v).
3. Add BHQ-1 carboxylic acid (0.5 mg, 1 μmol), triethylamine (0.1 mmol), and DMT-MM (29.5 mg, 0.1 μmol) in 0.5 mL of dioxane–H_2O (3:1, v/v) to the drained resin, and shake the suspension at room temperature overnight (*see* **Note 1**).
4. Drain the coupling solution from the resin, and add the freshly prepared acetic acid (30 mg, 0.5 mmol) and DMT-MM (147.4 mg, 0.5 mmol) in 0.5 mL of dioxane–H_2O (3:1, v/v) to block unreacted amino groups on the resin.
5. After overnight incubation, drain the resin and wash thoroughly with dioxane–H_2O (1:1, v/v) followed by methanol–H_2O (1:1, v/v).
6. Suspend the resin with three bed volumes of methanol–H_2O (1:3, v/v) to make a 25 % slurry. Store at –20 °C until use.

Table 1
Reaction mixture for PCR amplification of DNA pools

Template DNA[*1]	10 μL
10× *Pfx* amplification buffer	50 μL
10 mM dNTP mixture	15 μL
50 mM $MgSO_4$	10 μL
Forward primer[*2]	5 μL
Reverse primer[*3]	5 μL
Platinum *Pfx* DNA polymerase	10 μL
d.D.W	395 μL
Total	500 μL

3.2 In Vitro Selection Procedures

In vitro selection was performed to isolate RNA aptamers for BHQ-1 according to the standard procedure (Fig. 1).

3.2.1 Preparation of DNA Pools

1. Assemble the reaction mix in PCR tubes as described in Table 1 (*see* **Note 2**). [*1]Template DNA: 5′-GAA TTC CGC GTG TGC ACA CC-N60–GTC CGT TGG GAT CCT CAT GG-3′ (for preparation of first DNA pool a reverse-transcribed product was used for the other selection round). [*2]Forward primer: 5′-GCT AAT ACG ACT CAC TAT AGG GAA TTC CGC GTG TGC ACA CC-3′ (T7 promoter sequence is underlined). [*3]Reverse primer: 5′-CCA TGA GGA TCC GAA CGG AG-3′.
2. Place the tubes into a thermal cycler and perform the PCR amplification using the following program: 2-min initial denaturation at 94 °C, followed by sequential cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 68 °C for 1 min, and final extension at 68 °C for 7 min (*see* **Note 3**).
3. Precipitate the DNA by adding a half volume of 7.5 M ammonium acetate solution and 1.5 volume of isopropanol. Centrifuge the sample at 10,000 × *g* for 30 min to pellet the DNA.
4. Remove the supernatant carefully, and rinse the pellet with 70 % ethanol.
5. Air-dry the pellet for 5 min, and dissolve it in 50 μL of RNase-free water.

3.2.2 Preparation of RNA Pools

1. Assemble the transcription reaction at room temperature in the order shown below (Table 2).
2. Incubate the reaction mix at 37 °C overnight.
3. Add 10 μL of DNase I and incubate for 30 min at 37 °C.

Table 2
Reaction mixture for in vitro transcription of reaction

Template DNA	*X* μL
10× T7 RNA polymerase buffer	50 μL
NTP mixture (25 mM each)	15 μL
50 mM DTT	10 μL
T7 RNA polymerase	5 μL
RNase-free water	420 – *X* μL
Total	500 μL

4. Precipitate the RNA by adding a half volume of 7.5 M ammonium acetate solution and 1.5 volume of isopropanol. Centrifuge the sample at 10,000 × *g* for 30 min at 4 °C to pellet the RNA.
5. Thoroughly remove the supernatant, and dissolve the pellet in 0.5 mL of RNase-free water.
6. Apply the sample to the pre-equilibrated NAP-5 column, and elute the sample with 1 mL of RNase-free water (*see* **Note 4**).
7. Precipitate the RNA by adding 0.5 mL of 7.5 M ammonium acetate solution and 1.5 mL of isopropanol. Centrifuge the sample at 10,000 × *g* for 30 min at 4 °C to pellet the RNA.
8. Remove the supernatant carefully, and rinse the pellet with 70 % ethanol.
9. Air-dry the pellet for 5 min, and dissolve it in 100 μL of the annealing buffer.
10. Denature the RNA pool for 3 min at 80 °C, and then let it fold by slow cooling to room temperature (*see* **Note 5**).
11. Add 2 μL of $MgCl_2$ solution to a final concentration of 5 mM (*see* **Note 6**).

3.2.3 Binding of RNA Pool to BHQ-Immobilized Resin

1. Place 50 μL of the BHQ-1-immobilized resin (25 % slurry) into an empty column (Micro Bio-Spin Chromatography Columns) (*see* **Note 7**), and wash the resin three times with 150 μL of the binding buffer.
2. Apply the RNA pool solution to the resin and incubate for 30 min on ice. Vortex the sample briefly every 10 min.
3. Drain and wash the resin to remove the unbound RNA species (*see* **Note 6**).
4. Elute bound RNA species three times with 130 μL of the binding buffer saturated with free BHQ-1 amine (*see* **Note 7**).

Table 3
Reverse transcription reaction

a	
Template RNA	8 μL
10 mM dNTP mix	1 μL
10 μM reverse primer*	1 μL
Total	10 μL
[*Reverse primer: 5′-CCATGAGGATCCGAACGG AC-3′]	
	↓ 65 °C, 5 min
b	
Template RNA/primer mixture	10 μL
5× PrimeScript buffer	4 μL
RNase inhibitor	0.5 μL
PrimeScript reverse transcriptase	1 μL
RNase-free water	4.5 μL
Total	20 μL
	↓ 42 °C, 60 min, then 70 °C, 15 min

5. Collect the eluate in a 1.5 mL collection tube, and add 43 μL of 3 M sodium acetate solution and 1 mL of ethanol to precipitate recovered RNA. Centrifuge the sample at 10,000 × *g* for 30 min at 4 °C.
6. Remove the supernatant carefully, and rinse the pellet with 70 % ethanol.
7. Air-dry the pellet for 5 min, and dissolve it in 8 μL of RNase-free water.

3.2.4 Reverse Transcription

1. Prepare the following mixture (Table 3a) in a PCR tube. Heat the sample for 5 min at 65 °C and then chill immediately on ice. Assemble the reaction mix as shown in Table 3b, and perform cDNA synthesis reaction for 60 min at 42 °C, followed by heat-inactivation of RTase at 70 °C for 15 min.
2. Use 10 μL of the reverse-transcribed product as a template for the PCR amplification to generate the DNA pool for the next round of selection (*see* **Note 8**).
3. Repeat the selection steps for several rounds to enrich the RNA species with high affinity to BHQ-1.

3.3 Cloning and Sequencing of Each RNA Species in the Enriched Pool

1. After the final round of selection, digest the resulting DNA pool with *Eco*RI and *Bam*HI as well as pUC19.
2. Ligate the digested insert and vector using DNA Ligation Kit.
3. Transform DH5α with the ligation product, and heat shock and spread the cells on LB plate containing 100 μg/mL of ampicillin. Incubate the plate at 37 °C overnight.
4. Culture from a single colony, and isolate the plasmid using standard plasmid isolation procedures.
5. Sequence the insert using primers in the vector backbone.

3.4 Fluorescence Measurements

To determine if the selected aptamers have the ability to restore the fluorescence of the fluorophore–BHQ-1 conjugates, fluorescence titration of the fluorophore–BHQ-1 conjugates with the aptamers can be performed. The synthesis of the fluorophore–BHQ-1 conjugate was described in our previous report [14].

1. Prepare the dsDNA template containing T7 promoter and each BHQ-1 aptamer by PCR amplification using the individual clone as a template. Primers used in the reaction are as follows: the forward primer [5′-GCT AAT ACG ACT CAC TAT AGG GAA TTC CGC GTG TGC ACA CC-3′ (T7 promoter sequence is underlined)] and the reverse primer [5′-CCA TGA GGA TCC GAA CGG AC-3′].
2. Perform a transcription reaction, and purify RNA as described above in Subheading 3.2.2.
3. Prepare a solution of conjugate AL at the concentration of 2 μM in the binding buffer.
4. Prepare RNA solution at the concentrations of 0, 0.6, 2, 6, 20, 60, and 200 μM in the binding buffer.
5. Mix an equal volume of the RNA solution with the conjugate solution, and measure the fluorescence of each sample using either a plate reader or a fluorophotometer. The excitation maximum of each conjugate and the emission maximum in the RNA-bound state are as follows: conjugate AL (Ex/Em = 543 nm/610 nm), conjugate Cy (Ex/Em = 519 nm/562 nm), and conjugate FL (Ex/Em = 497 nm/515 nm).

The K_d values of each aptamer can be obtained by fitting fluorescence titration data using the following equation:

$$F_{obs} = A\left(\frac{\left(\left[conjugate\right]_T + \left[aptamer\right]_T + K_d\right) - \left(\left(\left[conjugate\right]_T + \left[aptamer\right]_T + K_d\right)^2 - 4\left[conjugate\right]_T\left[aptamer\right]_T\right)^{1/2}}{2\left[conjugate\right]_T}\right)$$

Table 4
In transcription/translation reaction mixture

		Final conc.
Template DNA	XμL	0.5 μg
E. coli lysate	4.8 μL	
Reaction mix	4 μL	
Amino acids	4.8 μL	
Methionine	0.4 μL	
Reconstitution buffer	2 μL	
100 μM fluorophore–BHQ-1 conjugate	0.5 μL	2.5 μM
RNase-free water	3.5 – XμL	
Total	20 μL	

where A is the increase in fluorescence at saturating aptamer concentrations ($F_{max} - F_{min}$), K_d is the dissociation constant, and $[\text{conjugate}]_T$ and $[\text{aptamer}]_T$ are the total concentrations of a conjugate and an aptamer, respectively.

3.5 Real-Time Monitoring of Protein Synthesis

Detailed protocol of the preparation of template DNAs is described in our previous report [14].

1. Assemble the reaction according to Table 4.
2. Incubate the reaction mixture at 30 °C, and collect the fluorescence emission data every 3 min at appropriate wavelength by using a real-time PCR system with the filter for FAM and ROX dye.
3. After 1-h incubation, add DNase I to the mixture and incubate for 30 min at 37 °C to digest the template DNA.
4. Analyze the resulting reaction to verify RNA and protein synthesis by RT-PCR and western blotting, respectively.

4 Notes

1. We use DMT-MM as a coupling reagent, but any other water-soluble coupling reagents such as EDC can be used.
2. For the preparation of first DNA pool, use a sufficient amount of template DNA to minimize the loss of individual sequences in the random library.
3. Perform a cycle course PCR amplification to determine the relative quantities of the bound RNA species using one-tenth aliquot of the reaction mixture. For each sample, 4 μL of aliquots

are removed from the reaction after 4, 8, 12, 16, 20, 24, 28, and 32 cycles and separated on an 8 % native polyacrylamide gel. As the population of binding species becomes enriched, a band of the desired product will appear at an earlier PCR cycle than the previous selection round.

4. We use NAP-5 column to remove unincorporated NTPs, but any other gel filtration system can be used.
5. Do not heat the RNA solution in the presence of Mg^{2+}. This is important to avoid possible chemical degradation of RNA with divalent cations.
6. We usually measure the UV absorbance of the RNA pool solution before and after binding to the resin to determine the percentage of bound RNA in each cycle. The percentage of RNA bound to the resin is calculated as 100(AbsT – AbsF)/AbsT, where AbsT is UV absorbance of the RNA solution before binding to the resin, and AbsF is UV absorbance of the flow-through fraction after binding to the resin.
7. Do not use a spin column with a microporous membrane such as PVDF, because BHQ-1 amine may adsorb to the membrane, probably due to the hydrophobicity of BHQ-1.
8. We usually save a half of the reverse-transcribed product for archival purposes. If subsequent amplification or selection reactions fail for any reason, the procedure can be started from the archival pool.

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