

Identification of Aptamers as Specific Binders and Modulators of Cell-Surface Receptor Activity

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Abstract

In recent years, the SELEX (Systematic Evolution of Ligands by EXponential enrichment) technology has established itself as a powerful tool in basic research with promising applications in diagnostics and therapeutics. Oligonucleotides with high-affinities to their targets, denominated as aptamers, are obtained from partially random oligonucleotide pools by reiterative in vitro selection cycles and screening for binding activity. The original technique allowing the identification of aptamers binding to soluble targets, has recently been extended in order to produce aptamers binding to complex targets including receptors and ion channels embedded in the plasma membrane as well as whole cell surfaces or parasite organisms. In addition to discussing the most recent developments with focus on possible diagnostic and therapeutic application, we provide a simple protocol which has been successfully used to select for RNA aptamers as allosteric modulators of nicotinic receptor activity.

Key words: RNA aptamers, SELEX, Nicotinic acetylcholine receptors

1. Introduction

The SELEX (Systematic Evolution of Ligands by EXponential enrichment) was introduced in 1990 by Larry Gold and Jack Szostak (1, 2) which has been evolved into an extremely potent technology to isolate high-affinity oligonucleotide ligands and inhibitors from random oligonucleotide libraries of up to 10^{16} different molecules and possible secondary and tertiary structures. The isolated molecules were named aptamers (from aptus = latin to fit). In the beginning, RNA and DNA aptamers were identified which bind to proteins that naturally interact with nucleic acids or small molecules such as ATP. In the following years, the use of the SELEX technique was extended in order to isolate oligonucleotide ligands for a wide range of proteins of importance for therapy and diagnostics, such as growth factors (3), cell-surface antigens, entire

cells, and even whole organisms (4–6). Since these RNA and DNA molecules bind their targets with similar affinities and specificities as antibodies, and are able to distinguish between isotypes of an enzyme, aptamers have been also called synthetic antibodies (7). Recently, the use of in vitro selection methods has been extended to complex targets, such as receptors that are only functional in their membrane-bound form (8), and living organisms such as bacteria (9), trypanosomes (10), and *Plasmodium falciparum*-infected erythrocytes (11). Moreover, since it became apparent that aptamers are capable to distinguish between little differences in cell-surface marker proteins expressed by, i.e., cancer cells, parasite-infected cells, or stem-cells in comparison to normal somatic cell (5), they have been developed into promising agents for diagnostic and therapeutic applications.

Aptamers are ideal candidates for studying protein interactions in vitro and in vivo and for developing novel molecules of pharmaceutical importance for the following reasons: aptamers isolated from combinatorial library have high dissociation constants, ranging from nanomolar to femtomolar, similar to the best affinity interactions between monoclonal antibodies and antigens. Most importantly, the aptamers can be chemically altered depending on their desired purpose. Chemical modifications that enhance aptamer stability for in vivo applications and possible therapy include substitutions of the 2'OH group of the ribose backbone of pyrimidines by amino, fluoride, or O-methylene functions. With such modifications, the half-life of RNA aptamers in biological fluids such as human serum or urine increases from approximately 8 s for unmodified RNA to 86 h for fluoro- or amino-modified RNA (12). Modifications at the 5' position of pyrimidines expand the possibilities for chemically decorating oligonucleotide libraries including iodide or bromide substitutions or the introduction of SH-groups for chemical and photocross-linking of aptamers with their targets. Aptamers can be attached to radioactive or fluorescent molecules and can be used this way for in vitro and in vivo imaging of target proteins.

Aptamers can be selected against any proteins within a complex mixture of potential targets and then be used for ligand-mediated target purification. Larry Gold and coworkers developed aptamers against erythrocyte membranes that bind specifically to prior unidentified target proteins (4). The same strategy, also denominated as deconvolution SELEX, was used to stain rat brain tumor vessels and to identify the endothelial regulatory protein pigpen as aptamer-target protein (13). These studies indicate the possibility of purifying any protein target for which a high-affinity nucleic acid ligand has been identified. In order to generate covalent cross-links of selected aptamers with their targets, pyrimidines of the oligonucleotides are modified at the C5 position of the ribose. The aptamers can either be selected from oligonucleotide libraries that contain

photo-cross linkable pyrimidines or already selected aptamers can be modified posterior to the SELEX process. These modified oligonucleotides attached to a reporter molecule generate reactive groups that, when irradiated by UV light, can form covalent linkage with another molecule in close proximity. For ligand-mediated target purification, aptamers are selected that recognize specifically an unknown target protein, i.e., in a cell membrane. Binding specificity is based on the fact that a given aptamer species (i.e., radiolabeled) can be eluted by a competitor (molar excess of unlabeled vs. labeled aptamer) from the cell membrane. In a second step, the 5'-biotin-labeled aptamer is immobilized on a solid surface, such as streptavidin-coated magnetic beads, and then used as an affinity matrix to purify and identify the bound target protein. The binding proteins retained on aptamer-coated magnetic beads are separated from the remaining protein mixture by applying a magnetic field. Alternatively, aptamers can be covalently photo-cross-linked to their targets and used as a tracer in order to separate the target protein from remaining proteins in a cell extract.

Aptamers are capable to identify individual epitopes on cell surfaces as it has been demonstrated for human membranes. Since aptamers differentiate in molecular signature of cell-surface antigens, this technology has been recognized as a powerful tool for biomarker discovery and targeting cancer cells. Aptamer probes were developed recognizing leukemia, prostate, or glioblastoma cancer cells (14–16). Molecular recognition is a key mechanism for the diagnosis of cancer which has been exploited by applying multiple anticancer cell aptamers conjugated on nanorods. Up-regulated ErbB2 expression in breast cancer cells was targeted by fluorescence-tagged RNA aptamers in surface plasmon resonance measurements (17). Aptamer-based therapeutic approaches are based on the specific delivery of si-RNAs or toxic loads to tumor cells (18, 19) or by inhibition of cancer cell-surface receptors, such as the EGF receptors involved in protection of tumor cells against apoptosis (20, 21).

Moreover, aptamers have been developed as specific ligands and/or inhibitors of neurotransmitter receptors, including GABA receptors, AMPA-glutamate receptors, as well as neuronal and nicotinic acetylcholine receptors (nAChRs) (22–25, 8). In addition to acting as inhibitors, such as anesthetics, anticonvulsives, or the abused drug cocaine, on nAChRs, aptamers can be developed as specific ligands of defined conformational sites of neurotransmitter receptors in order to reestablish dysfunctions in channel-open and -closing equilibrium. Such approaches aim at reestablishment of decreased GABA A-receptor activity in epilepsy involving mutation of this receptor (22), or at restoring of normal nicotinic receptor activity following inhibition by abused drugs such as cocaine and phencyclidine (23).

A simple protocol detailed in this chapter was established to develop RNA aptamers that bind to the electric organ nAChR and that are displaced by cocaine (8) (see Fig. 1 for a scheme). This protocol can be easily transferred to SELEX applications with other receptors or cell-surface epitopes, given that these are enriched in membrane preparations.

The aptamers obtained against the nAChR either are biologically active by themselves and inhibit the muscle-type nAChR activity as cocaine does or are by them biologically inactive and, therefore, protect the receptor against inhibition by cocaine (25).

Obtaining RNA aptamers by using the SELEX technology is a conceptually straightforward process. In the beginning, an oligonucleotide is synthesized by a DNA synthesizer that consists of a random sequence of typically 16–75 oligonucleotides flanked by two constant regions. A T7-promoter site is incorporated in one of the constant sequences by taking advantage of the introduced EcoRI and HindIII sites, which allows removal of the constant sites and to insert the random regions into the multi-cloning site of a bacterial vector. Random sequences are created by premixing all four nucleotides during DNA synthesis. The double-stranded DNA template is enzymatically generated and amplified by primer extension and error-prone PCR. The random nucleotide sequence of the double-stranded DNA template is verified by cloning and sequencing of around 40 individual RNA molecules. In the random sequence of these RNA molecules, sequence motifs (i.e., AA, AC, AG, AT) should be equally distributed.

For the *in vitro* selection process, the RNA pool containing 10^{13} different sequences and structural motifs is generated by an *in vitro* transcription reaction. Folding of the RNA molecules is induced by heat denaturation and renaturation at room temperature (26).

The RNA molecules are made nuclease resistant either by incorporating modified bases, i.e., 2'-fluoro- or by employing 2'-amino-modified pyrimidines during the *in vitro* transcription step (12), or by protection during the selection step using a dithiothreitol (DTT)-independent RNase inhibitor (27). The RNA pool is exposed to its target in a 10–1,000 ratio of RNA:target-binding sites, and ligands are separated from nonfunctional molecules by filtration or by migration on polyacrylamide gel where target-bound RNA molecules migrate more slowly than unbound RNA which allows gel purification as indicated in Fig. 2 (28).

The recovered RNA is reverse transcribed to cDNA and amplified. The next-generation RNA pool, generated again by *in vitro* transcription, is already enriched in RNA molecules that bind to their target. The procedure is repeated with rising stringency until the random RNA pool is purified to a fraction of RNA

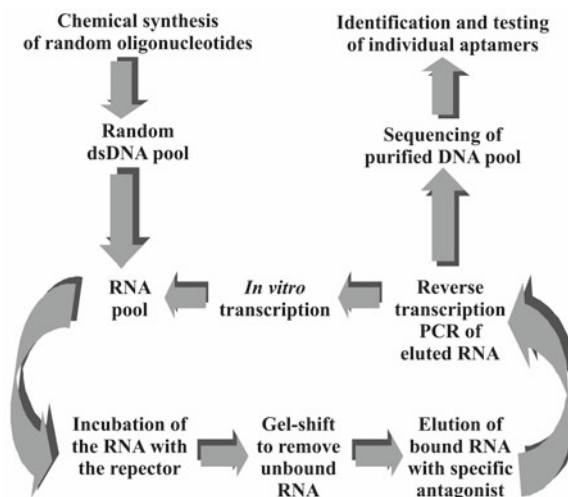


Fig. 1. Scheme for RNA aptamer identification targeting cocaine-binding sites on the nicotinic acetylcholine receptor (nAChR).

molecules with the desired binding properties and no further improvement of binding can be achieved. The final RNA pool is reverse transcribed into cDNA, amplified by PCR, and cloned into a bacterial vector. Clones containing the aptamer insert are picked and about 40 individual aptamers are identified by sequencing. The previously random regions are aligned and analyzed for consensus motifs, and the structures of RNA aptamers containing consensus motifs are predicted on the basis of free energy minimization (29). Aptamers containing consensus motifs are characterized regarding their binding affinity for their target and are screened for their biological activity by patch-clamping (30), using the whole-cell current-recording technique (31) in combination with rapid ligand delivery within 10 ms (called the cell-flow technique) (32, 33) (see Fig. 2.1 for a general scheme of RNA aptamer selection).

The protocol described here for the nAChR can be used with modification for the selection of aptamers that bind to other proteins expressed on a cell surface. In addition to the electrophysiological assay described here, screening for biological activity can include the quantification of ion flux into cells using fluorescent reporters (34, 35). For imaging purposes, fluorescence-tagged nucleotides can be enzymatically incorporated into the aptamer sequence (i.e., biotin-CTP or fluorescein-CTP). Alternatively, the aptamer can be modified at its 5'-end in order to attach a biotin moiety by substituting 5'-GTP for 5'-GDP- β -S and followed by covalent coupling of biotin molecule to this group and addition of a streptavidin-fluorophore (12, 36).

2. Materials

2.1. Target Preparation

1. Motor-driven blade homogenizer, low-speed refrigerated centrifuge, ultracentrifuge, spectrophotometer, and gamma counter.
2. Frozen electric organ from *Torpedo californica* (Aquatic Research Organisms, Inc., Hampton, NH): Maintain at -80°C .
3. Buffer H: 10 mM sodium phosphate, 400 mM sodium chloride, 5 mM Na_2EDTA , 10 mM sodium azide, at pH 7.4. Per liter: 2.68 g $\text{Na}_2\text{HPO}_4 \times 7\text{H}_2\text{O}$, 112.88 g NaCl, 1.86 g Na_2EDTA , 0.65 g NaN_3 . Adjust to pH 7.4 with 1 M HCl.
4. Buffer A (buffer H without NaCl).
5. Phenylmethylsulfonylfluoride (PMSF) 0.1 M stock solution: 0.174 g PMSF in 10 ml isopropanol.
6. 60% (w/w) sucrose solution. Per 100 ml: 77.2 g D-glucose in buffer A.
7. 36% (w/w) sucrose solution. Per 100 ml: 60 ml 60% sucrose solution diluted to 100 ml with buffer A.
8. Bovine serum albumin and Lowry protein determination reagents.
9. Buffer B: 10 mM sodium phosphate, 50 mM NaCl, 0.1% (w/v) Triton X-100 at pH 7.5. Per liter: 2.68 g $\text{Na}_2\text{HPO}_4 \times 7\text{H}_2\text{O}$, 14.11 g NaCl, 1.0 ml Triton X-100. Adjust to pH 7.5 with 1 M HCl.
10. Unlabeled α -bungarotoxin (BGT) solution: Prepare a 10 mM BGT stock solution from lyophilized BGT (Sigma, St. Louis, MO).
11. ^{125}I -BGT solution: Prepare a 200 nM ^{125}I -BGT stock solution from lyophilized ^{125}I -BGT (Perkin Elmer/New England Nuclear, Boston, MA).
12. DE-81 filters (Whatman, Kent, UK).

2.2. SELEX Procedure

1. Equipment: PCR machine, scintillation counter, tabletop centrifuge, temperature-controlled water baths, equipment for horizontal and vertical electrophoresis, UV-illuminator, phosphor imager, automatic DNA sequencer, vacuum dot-blot manifold (Schleicher and Schuell). PCR 0.5 ml hot-start tubes, aerosol resistant pipette tips, autoclaved Eppendorf tubes (all from Fischer Scientific, Brightwaters, NY) and glassware, diethyl pyrocarbonate (DEPC, Sigma)-treated solutions.
2. Sterile disposable Petri dishes and sterile inoculating loops for bacterial culture.
3. BA-85 nitrocellulose and gel-blotting paper (GB002, both 102×133 mm, Schleicher and Schuell, Keene, NH).

4. GF/F glass fiber filters, 1.3 cm diameter (Whatman, Kent, UK).
5. Sterile syringe filters (0.45 μm; 25 mm diameter) (Whatman).
6. Sigmacote (Sigma).
7. Kodak X-Omat K Diagnostic Film for autoradiography or imaging plate for phosphor imager.
8. Incubation buffer: 25 mM HEPES, 145 mM sodium chloride, 5.3 mM potassium chloride, 1.8 mM calcium chloride dihydrate, 1.7 mM magnesium chloride hexahydrate, pH 7.4. Per 1 l: 59.5 g HEPES, 8.47 g NaCl, 3.95 g KCl, 2.65 g CaCl₂ × 2H₂O, 3.46 g MgCl₂ × 6H₂O. Adjust to pH 7.4 with 1 N NaCl.
9. pGEM-3Z vector (cloning vector that allows highly efficient synthesis of RNA in vitro) (Promega, Madison, WI) and for transformation of *E. coli* competent cells (strains JM 29 or DH5α) (Promega).
10. pCR4-TOPO vector with One-shot cells (for fast cloning) (Invitrogen, Carlsbad, CA).
11. LB medium and agar: Per liter LB medium dissolve in distilled water (dd H₂O) and autoclave: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 ml 1 N NaOH. Per liter LB agar dissolve in dd H₂O and autoclave: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 ml 1 N NaOH, 15 g agar.
12. Partial random DNA template (108 bp) (synthesized by Biosource International, Foster City, CA).
5'-ACC-GAG-TCC-AGA-AGC-TTG-TAG-TAC-TNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-CCT-AGA-TGG-CAG-TTG-AAT-TCT-CCC-TAT-AGT-GAG-TCG-TAT-TAC-3' (N=A, C, G, or T are incorporated with equal probabilities). Restriction sites (HindIII and EcoRI) are underlined.
13. Primer region 1 (P-40, 40 bp, forward primer with T7 promoter site)
5'-GTA-ATA-CGA-CTC-ACT-ATA-GGG-AGA-ATT-CAA-CTG-CCA-TCT-A-3'.
14. Primer region 2 (P-22, 22 bp, reverse primer).
5'-ACC-GAG-TCC-AGA-AGC-TTG-TAG-T-3'
Reverse primer for aptamer amplification in pGEM-3Z vector (P-22 pGEM, 22 bp)
5'-GAA-TAC-TCA-AGC-TTG-TAG-TAC-T-3' (forward primer is the same as above).
15. Kits and enzymes: Superscript reverse transcriptase (Invitrogen), Maxiscript and Megascript in vitro transcription kits (Ambion, Austin, TX). Taq-DNA polymerase, T4-polynucleotide kinase, EcoRI and HindIII restriction enzymes (Invitrogen).

16. Wizard Miniprep kit (Promega).
17. Big Dye sequencing kit (Applied Biosystems, Foster City, CA).
18. DTT-dependent and -independent RNase inhibitors (Ambion), NTPs, dNTPs, MgCl_2 , MnCl_2 , yeast t-RNA, DNA, and RNA molecular weight standards.
19. Acrylamide/bisacrylamide, low-melting agarose (Sea Plaque GTG, Biocompare, Inc, South San Francisco, CA).
20. Ethanol, saturated phenol (pH 7 and 5.2), chloroform, iso-amylalcohol (molecular biology grade, Sigma).
21. Tris-borate-EDTA (TBE) buffer (10 \times solution): 0.89 M TRIZMA base, 0.89 M H_3BO_3 , 20 mM EDTA. Per liter: 108 g TRIZMA base, 55 g boric acid, 40 ml 0.5 M ethylenediaminetetraacetate (EDTA) solution.
22. Tris-EDTA (TE) buffer: 10 mM TRIZMA hydrochloride, 1 mM EDTA.
10 mM Tris-HCl, 1 mM EDTA, pH 7.5. Per liter: 1.57 g Tris-HCl, 0.29 g EDTA. Adjust to pH 7.5.
23. Formamide-loading buffer for RNA gel: Per 10 ml add 0.2 ml 0.5 M EDTA, pH 8.0, 10 mg bromophenol blue, 10 mg xylene cyanol, 10 ml formamide (deionized).
24. Loading buffer for DNA gel: Per 10 ml: 1 ml 10 \times TBE, 10 mg bromophenol blue, 10 mg xylene cyanol, 2 g glycerol.
25. Urea, ammonium acetate, sodium acetate.
26. Spin columns S-30 (cut-off 30 nucleotides) (Sigma).
27. Cocaine hydrochloride (1 mg/ml in methanol) (Sigma): Evaporate the methanol and resuspend in incubation buffer at a concentration of 10 mM.
28. MK-801 (RBI Biochemicals, Natick, MA): Dissolve at a concentration of 10 mM in incubation buffer.
29. (α - ^{32}P)-UTP (3,000 Ci/mmol), (γ - ^{32}P)-ATP (5,000 Ci/mmol), both from Amersham Biosciences, Uppsala, Sweden.

2.3. Cell Culture and Electrophysiology

1. Equipment: CO_2 -cell culture incubator, laminar flow cabinet, vibration-isolation table (TMC, Peabody, MA), Faraday cage (TMC), Axopatch 200B integrated patch-clamp amplifier with Digidata 1322A interface and pClamp program (Axon Instruments, Union City, CA), inverted microscope, one micro manipulator and two coarse manipulators (Narishige International, East Meadow, NY), computer-operated 12 V solenoid valves (Lee Company, Westbrook, CT), two peristaltic pumps for ligand and inhibitor delivery (Rainin, Oakland, CA), Kendall tubing diameter: 0.05, 0.16, and 0.42 cm (VWR International, Westchester, PA), U-shaped stainless steel capillary tubes (250 μm inner diameter) with a circular porthole of

150 mm at the base of the U (37) for ligand and inhibitor application made from Hamilton HPLC-tubing (Hamilton Corp., NV), patch-pipette puller (Sutter, Novato, CA), pipette polisher (Microforge, Narishige International, USA).

2. BC₃H1 cells (American Type Culture Collection, Manassas, VA).
3. DMEM high glucose, trypsin-EDTA solution, fetal bovine serum (FBS), heat inactivated (all from Invitrogen).
4. 25 cm² cell culture flasks (Costar, Corning, Acton, MA).
5. 15 ml screw capped vials (Greiner, Fischer Scientific).
6. 35 mm cell culture dishes (Corning, Acton, MA).
7. Incubation buffer (extracellular buffer): 25 mM HEPES, 145 mM NaCl, 5.3 mM KCl, 1.8 mM CaCl₂ × 2H₂O, 1.7 mM MgCl₂ × 6H₂O, pH 7.4.
8. Pipette solution (intracellular buffer): 25 mM HEPES, 140 mM potassium chloride, 10 mM sodium chloride, 2 mM magnesium chloride hexahydrate, 1 mM EGTA, pH 7.4. Per liter: 59.5 g HEPES, 10.44 g KCl, 0.58 g NaCl, 0.41 g MgCl₂ × 6H₂O, 0.38 g EGTA. Adjust to pH 7.4 with 1 M KCl.
9. Carbamoylcholine (Sigma).
10. Cocaine hydrochloride (Sigma).
11. Borosilicate glass capillaries for patch pipettes (World Precision Instruments, Sarasota, FL).

3. Methods

3.1. Preparation and Evaluation of the nAChR-Enriched Plasma Membranes as a Target for the In Vitro Selection Process

1. Weigh 60–80 g of frozen electric organ and return it to the freezer. Measure a volume of buffer H equal in ml to the tissue weight in grams and add PMSF to a final concentration of 1 mM. Put the frozen pieces of organ in a sturdy cloth sack and pulverize with a hammer. Transfer the still frozen powder into a graduated cylinder, add the buffer containing PMSF, and leave the tissue/buffer mixture for about 10 min at room temperature to thaw. Then homogenize the tissue for 30 s four times using a motor-driven blade homogenizer at maximal speed, resting 10 s between each run. All subsequent steps should be performed on ice.
2. Transfer the homogenate into precooled centrifuge bottles and centrifuge for 10 min at 2,400 × g, 4°C. Collect the supernatant and discard the pellet. Transfer the supernatant to a precooled ultracentrifuge bottle and ultracentrifuge for 90 min at 34,000 × g, 4°C. Discard the supernatant and resuspend the

pellet in 60 ml of buffer A containing 1 mM PMSF by homogenizing the pellet three times for 10 s each using a motor-driven blade homogenizer at low speed. Wash the pellet again as above in buffer A and resuspend the pellet in 25 ml of buffer A containing 1 mM PMSF.

3. This step employs a discontinuous gradient ultracentrifugation method (38). Add 44 ml of 60% sucrose solution to the resuspended pellet, mix well, transfer to a 250 ml graduated cylinder, and add buffer A until the total volume is 108 ml to obtain a final sucrose concentration of 28%. Add 10 ml of a 36% sucrose solution to six swinging bucket rotor 30 ml ultracentrifuge tubes. Gently overlay the layers in each centrifuge tube with 18 ml of the resuspended pellet. Top off each tube with buffer A. Ultracentrifuge on a swinging bucket rotor for 4 h at $100,000 \times g$, 4°C . Aspirate and discard the floating pad at the buffer A/28% sucrose interface, together with most of the 28% layer. Collect the visible membrane layer containing nAChRs at the 28%/36% interface. Dilute the recovered 28%/36% interface with an equal volume of buffer A and collect the pellet by ultracentrifugation for 30 min at $38,000 \times g$, 4°C . Resuspend the pellet in 10 ml buffer A using a syringe equipped with a #18 needle. Aliquot and store the membranes frozen at -80°C .
4. The concentration of the membrane proteins is determined according to the method of Lowry et al. (39) using bovine serum albumin as the reference standard.

3.2. Determination of nAChR-Binding Sites in Torpedo Electric Organ Membrane Preparations

(^{125}I) BGT binding to the receptor is assayed using a filtration method (40).

1. Dilute 50 μl of the membrane suspension 1:100 in buffer B and then measure 50 μl aliquots of the diluted membranes into six Eppendorf vials.
2. To each of the three vials add 50 μl buffer B and to each of the other three vials add 50 μl unlabeled BGT solution.
3. Vortex and preincubate on a rotator at room temperature for 60 min. Then add 100 μl of ^{125}I -BGT to each vial.
4. Vortex and incubate on a rotator at room temperature for 60 min.
5. Load vacuum manifold ports with DE-81 filters (Whatman).
6. Prewash filters with 0.6 ml buffer B using vacuum.
7. Transfer 50 μl of each reaction mixture directly onto the filters (do this in triplicate) and wash each filter with 1 ml buffer B.
8. Allow buffer to filter through and air-dry the filters for 1 min using vacuum suction.

9. Transfer filters into counting vials and determine filter-bound radioactivity in a gamma counter. Subtract nonspecific binding (binding with unlabeled BGT present) to determine the concentration of specifically bound ^{125}I -BGT. The concentration of nAChR in the membrane suspension is one-half this value ($\times 400$) since there are two BGT-binding sites per nAChR molecule.

3.3. Preparation of the Random DNA Pool for In Vitro Transcription

1. Check purity by resuspending 35 nmol of the pre-purified single-stranded oligonucleotide in 100 μl TE buffer. End label 1 μl of the oligonucleotides with $(\gamma)^{32}\text{P}$ ATP using T4-kinase and check for purity on 8% denaturing urea (6 M) PAGE (41). If there is not a single clean band, but shorter oligonucleotides are also present, the random oligonucleotides need to be purified.
2. Prepare a purification gel by adding an equal amount of formamide loading buffer to the resuspended DNA pool and a small amount of ^{32}P -labeled DNA from step 1.
3. Denature the mixture at 65°C for 10 min and load into nine lanes (each lane contains 500,000 cpm).
4. Expose gel using a phosphor imaging plate (15 min), and match the obtained image with the gel to localize the bands with the right size.
5. Cut out the bands and reimage the gel.
6. Crush the gel slices with a pipette tip and elute the DNA twice with 400 μl sodium acetate (0.3 M, pH 5.2) and 1 mM EDTA (for 12 h each).
7. Ethanol-precipitate the eluted purified DNA (see Note 1).

3.4. Second-Strand Synthesis and Amplification by Error-Prone PCR

Use hot-start tubes and assemble the bottom and top part of the reaction for second-strand synthesis and amplification of the DNA template by error-prone PCR. Hot-start PCR is the PCR technique of assembling the reaction mixture at a temperature that is greater than the annealing temperature. This procedure increases precision, yield, and specificity. The pre-adhered wax bead assures synchronous reaction start-up and eliminates the need for using mineral oil.

1. Assemble the reaction as reported in Table 1. This mix is enough for 30 PCR reactions of 120 μl volume each.
2. Second-strand synthesis: Execute the following program on a PCR machine:
 94°C 3 min, 42°C 2 min, and 72°C for 8 min. Then add 1.5 μl primer P-22 (100 pmol/ μl) to each 120 μl PCR and start the following program: 94°C 2 min, 42°C 1 min, and 72°C 1 min for one cycle and 94°C 2 min, 42°C 1 min, and 72°C 1 min + 0.5 min increase each round for 11 cycles.

Table 1
Second-strand synthesis and amplification by error-prone PCR mix

Addition	Bottom (μl)	Top (μl)
H ₂ O	981	1,540
10× PCR buffer	180	180
dATP (100 mM)	12	
dGTP (100 mM)	12	
dCTP (100 mM)	30	
dTTP (100 mM)	30	
MgCl ₂ (50 mM)	504	
P-40 (100 pM)	45	
ss DNA pool (60 pM)	6	
MnCl ₂ (1 M)		1.8
Taq DNA polymerase		30

3. Analyze on native PAGE (8% in 1× TBE buffer).
4. Repeat the protocol that is described above three times (so that the total reaction volume is 10.8 ml).
5. Purify if necessary.
6. Ethanol precipitate and dissolve in 100 μl dd H₂O.

3.5. Evaluation of the Degree of Randomness of the Pool

PCR amplification of the single-stranded (ss) DNA pool will result in multiple copies of a double-stranded DNA pool. Chemical lesions occurring during the chemical synthesis of the ss DNA pool, and the possibility that some sequences are more amplified than others during PCR procedures, may result in a limited pool size and predominance of sequence motifs in the random sequence (see below). Therefore it is necessary to sequence and analyze a small number of individual sequences of the random pool (2).

1. Clone 1 μl of the purified PCR product into a pCR4-TOPO vector and transform to “One Shot” competent cells (Invitrogen) according to the manufacturer’s recommendation.
2. Isolate and sequence 40 clones.
3. Use a word processing program to analyze random sequences for equal occurrence of AA, AC, AG, and AT motifs in the random regions (repeat for C, G, and T). If one or more of these motifs predominate or the base composition is not random in the random sequences, the pool may not contain enough different sequences for a successful SELEX experiment.

3.6. *In Vitro* Transcription and Purification of the SELEX RNA Pool

1. To perform *in vitro* transcription reaction: Save 20 μl of the total volume of 100 μl (for ^{32}P -UTP transcription for binding studies with nAChRs in *Torpedo* electric organ membranes, and in case you have to amplify the DNA again) and use 80 μl for transcription.
2. Assemble the following reaction: DNA solution (80 μl); H_2O (DEPC-treated) (74 μl); 10 \times synthesis buffer (25 μl); ATP, CTP, GTP, UDP 10 mM solutions (10 μl) each; DTT (100 mM) (6 μl); and T7 RNA polymerase (50 U/ μl) (25 μl).
3. Incubate the reaction at 37°C overnight.
4. Add 25 μl DNase (1 U/ μl) and incubate for 20 min.
5. Analyze 10 μl of the reaction product on a denaturing PAGE (8%, 6 M urea) to verify the right size of the RNA (90 nt) and to determine if smaller products are present.
6. If smaller reaction products are present, purify the full-length RNA from the gel to avoid the selection and amplification of shorter sequences. Alternatively, for generation of nuclease-resistant transcripts substitute CTP and GTP by 2'-fluoro- or 2'-amino-pyrimidines (TriLink BioTechnologies, San Diego, CA) at final concentrations of 1.2 mM.
7. For radiolabeling of the RNA pool, add 50 μCi (α - ^{32}P)-ATP (10 $\mu\text{Ci}/\mu\text{l}$).
8. To purify RNA, extract the reaction product with 275 μl of a saturated solution of phenol in 0.1 M NaOAc, pH 5.2.
9. The supernatant is then extracted with an equal volume of chloroform in order to remove remaining phenol and then ethanol-precipitated.

3.7. Selection of Aptamers that Bind to the nAChR and Displace Cocaine

The first two rounds are performed under low stringency conditions to enhance RNA–protein binding and to avoid early depletion of sequences present in the SELEX RNA pool. For SELEX cycles 1–3 a nitrocellulose-filter binding assay is used to separate receptor-bound from free aptamers. Beginning from SELEX cycle 4, the nitrocellulose-filter binding and a gel-shift selection step are employed as two consecutive selection processes (see Note 2).

1. The respective RNA pool is heated up for 10 min to 85°C prior to selection and allowed to cool down to room temperature in order to allow for proper formation of secondary and tertiary structures.
2. To perform SELEX experiment using nitrocellulose-filter binding, prepare filtration unit by preincubating a nitrocellulose sheet in incubation buffer.
3. Mount nitrocellulose sheet in autoclaved filtration unit. Assemble the following reaction mixtures for the SELEX process: nAChR-enriched plasma membranes (800 $\mu\text{g}/\text{ml}$

- protein; 1.6 μM receptor) (40 μl); incubation buffer (280 μl); anti-RNase (40 U/ μl) (40 μl); RNA pool (50 μM) (40 μl).
4. Incubate for 40 min at room temperature.
 5. Place 4 \times 100 μl of the selection mixture into each of the four wells of the filtration unit.
 6. Wash each well twice with 200 μl incubation buffer.
 7. Cut out the pieces of the nitrocellulose filters that cover the used wells of the filtration unit.
 8. For the elution of receptor-bound, cocaine-displaceable RNA aptamers, incubate each of these filter pieces in 100 μl of 1 mM cocaine (or 100 μl of 1 mM MK-801 that binds to the same site as cocaine on the nAChR) in incubation buffer for 20 min at room temperature.
 9. For the recovery and purification of the eluted RNA, add 5 μg t-RNA as a carrier, and phenol- and chloroform-extract the eluate, precipitate with ethanol, and resuspend the pellet in 12 μl DEPC-treated H_2O .
 10. Then perform reverse transcription (RT-PCR) assembling four separate reactions as follows: 2.5 μl of the recovered RNA, 2 μl P-22 (100 μM), and 6.5 μl DEPC-treated H_2O .
 11. Incubate at 70°C for 10 min, and then place on ice for 1 min.
 12. Add to the following RNA/P-22 solution: 14 μl RNA/P-22 solution, 2 μl 10 \times synthesis buffer, 2 μl dNTP mix (10 mM), 2 μl DTT (0.1 M), 1 μl MgCl_2 (50 mM), and 1 μl superscript RT (200 U/ μl).
 13. Incubate at room temperature for 10 min, then at 46°C for 50 min, and then at 70°C for 10 min.
 14. Place the vial on ice for 5 min, add 1 μl RNase H, and incubate at 37°C for 15–20 min (volume is now 21 μl).
 15. Transfer each reaction into a hot-start tube and prepare a PCR mix as reported in Table 2 and perform the following program: cycle 1 (94°C, 5 min; 60°C, 5 min; 72°C, 1 min), 5–10 cycles (94°C, 1 min; 60°C, 1 min; 72°C, 1 min), and final cycle (94°C, 1 min; 60°C, 1 min; 72°C, 10 min).
 16. Analyze 10 μl aliquot of the PCR product on a native PAGE (see Note 3).
 17. Gel-purify PCR products if necessary.
 18. Phenol- and chloroform-extract, ethanol-precipitate, and resuspend in 100 μl DEPC-treated H_2O .
 19. In vitro transcribe and purify RNA as already described.
 20. To perform gel-shift selection experiments, prepare a native gel, containing 3% acrylamide (stock: 38.5% acrylamide and 1.5% bis-acrylamide) and 1 \times TBE, adjusted to pH 7.4.

Table 2
PCR mix

Addition	Bottom (μl)	Top (μl)
RT-reaction product	21	
dd-H ₂ O	21	44
10× buffer	3	5
P-40 primer (50 μM)	2	
MgCl ₂ (50 mM)	3	
Taq polymerase (5 U/μl)		1

21. The RNA pool unlabeled or radiolabeled - as detailed later - is diluted in incubation buffer prior to the SELEX step, denatured and renatured as detailed in Subheading 3.7.
22. Assemble the following reaction: 20 μl nAChR-enriched plasma membranes (800 μg/ml protein; 1.6 μM receptor), 9 μl incubation buffer, 1 μl anti-RNase (40 U/μl), and 10 μl RNA (320 μM).
23. The reaction is carried out for 40 min at room temperature in incubation buffer.
24. Add one part of 4× loading buffer (20% glycerol, 0.2% bromophenol blue in 1× TBE) to three parts of reaction mixture.
25. Load the samples on the gel and electrophorese the gel at 10 V/cm for 3 h in 1× TBE, adjusted to pH 7.4.
26. Stain the gel with 0.5 μg/ml ethidium bromide.
27. Excise the band containing the RNA–protein complex.
28. Elute the bound RNA with 0.5 M NaOAc pH 5.2 containing 1 U/μl anti-RNase.
29. Phenol- and chloroform-extract and ethanol precipitate the recovered RNA (see Subheading 3.7). The RNA is reverse transcribed, purified, and precipitated as detailed in Subheadings 3.6 (25) and 3.7, and then RNA is used for the nitrocellulose filter selection step and cocaine displacement of nAChR-bound RNA molecules (Fig. 2).

3.8. Synthesis and Purification of ³²P-Labeled RNA

Radiolabeled RNA can be generated either by incorporation of a ($\alpha^{32}\text{P}$) nucleoside triphosphate during an in vitro transcription reaction or by the transfer of ($\gamma^{32}\text{P}$)-ATP to the 5' terminus of a dephosphorylated RNA molecule (41). The authors prefer the first mentioned method, as it needs only a single enzymatic reaction.

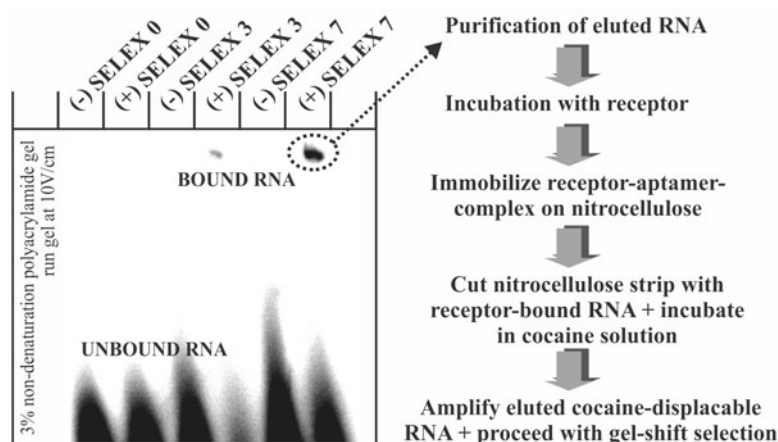


Fig. 2. Alternation of gel-shift and filter-binding selection steps: Target-bound and unbound radiolabeled RNA aptamers are separated by polyacrylamide gel electrophoresis, visualized by autoradiography, purified from the gel, and used for the subsequent nitrocellulose-filter binding selection step. The experiments are carried out in the presence (+) and absence (–) of target protein using the SELEX cycles 0 (control), 3, and 7. The figure illustrates the increase of binding affinity of selected RNA pools, seen as augmented quantity of RNA retained together with the receptor protein at the top of the gel (modified from ref. (8)).

1. Gel-purify DNA pools of various SELEX cycles.
2. Ethanol-precipitate the DNAs and resuspend at a concentration of 200 ng/ μ l for in vitro transcription reactions.
3. Make labelling cocktail: (Volume needs to be multiplied by the number of reactions to be set up). Add 6 μ l DEPC-treated H_2O , 2 μ l 10 \times transcription buffer, 1 μ l ATP (0.5 mM), 1 μ l CTP (10 mM), 1 μ l GTP (10 mM), and 1 μ l UTP (10 mM).
4. Add to the 12 μ l mix: 2 μ l DNA (SELEX pools), 5 μ l (α - ^{32}P)-ATP (10 μ Ci/ μ l), and 1 μ l T7 RNA polymerase (50 U/ μ l) for a total volume of 20 μ l.
5. Incubate at 37°C for at least 2 h.
6. Add 2 μ l DNase I (1 U/ μ l) and incubate for 15 min at 37°C.
7. Add 3 μ l 0.5-M EDTA and purify the ^{32}P -RNAs from unincorporated ^{32}P -nucleotides using a spin column 30 (Sigma).
8. Run an 8% denaturing gel (6 M urea) to check the size and the purity of the (^{32}P)-RNA (see Note 4). (For nuclease-resistant (^{32}P)-RNA aptamers set up transcription reactions in the presence of 1.5 mM 2'-fluoro- or 2'-amino-modified pyrimidines instead of CTP and UTP).
9. Gel-purify the (^{32}P)-RNA if necessary.
10. For gel purification of ^{32}P -labeled RNA, pour denaturing gel (8% acrylamide, 6 M urea) (see Note 3).

11. Load left and right outer lanes with loading dye, and pre-run the gel at 250 V until the dye has migrated down approximately 75% of the gel.
12. Mix (^{32}P)-RNA reaction mixture with 15 μl formamide loading buffer, and incubate at 85°C for 15 min.
13. Load samples and run the gel at 250 V until the rapidly migrating dye (bromophenol blue) has almost moved out of the gel.
14. When finished, visualize the location of the (^{32}P)-RNA on the gel by autoradiography or phospho imaging, and cut out the gel slices containing the full-length (^{32}P) RNA using a fresh, sterile razor blade for each sample.
15. Transfer the gel slabs into a 2 ml Eppendorf tube, crush with a pipette tip, and extract overnight with 1 ml 0.3 M NaOAc, pH 5.2, 10 μl DTT, and 5 μl RNAsin.
16. Pass the suspension through a syringe filter (0.45 μm sterile filter) to remove the gel, add an equal volume of a saturated solution of phenol in 0.3 M NaOAc pH 5.2, and vortex hard.
17. Centrifuge for 3 min at 12,000 $\times g$ in a tabletop centrifuge.
18. Collect the supernatant and concentrate it by ethanol precipitation (see Note 1).

3.9. Determination of Specific Binding to the nAChR

1. Soak GF/F filters (diameter 1.3 cm, Whatman) and a sheet of filter paper (GB002, 102 \times 133 mm, Schleicher and Schuell) for 1 h in incubation buffer containing 1% Sigmacote to reduce unspecific binding of the RNA molecules to the filters.
 2. The (^{32}P) RNAs are diluted in incubation buffer containing 10 mg/ml to a specific activity of 5×10^5 cpm/ μl and denatured and renatured before the experiment.
 3. The specific binding of the selected aptamers to their target is determined as the difference between the total binding and the unspecific binding in the presence of an excess of unlabeled competitor. Assemble the following reactions for duplicate estimations in a total volume of 100 μl (for determination of total and unspecific binding each). The specific binding is the difference between total and unspecific binding. Binding of the selected RNA molecules to the nAChR reaction: 89 μl incubation buffer, 10 μl nAChR-enriched plasma membranes (800 $\mu\text{g}/\text{ml}$ protein; 1.6 μM receptor), and 1 μl ^{32}P -RNA dilution containing 10 mg/ml t-RNA anti-RNase (40 U/ μl).
- Determination of unspecific binding reaction by competition of the RNA aptamers with cocaine: 79 μl incubation buffer, 10 μl nAChR-enriched plasma membranes (800 $\mu\text{g}/\text{ml}$ protein; 1.6 μM receptor), 1 μl ^{32}P -RNA dilution containing 10 mg/ml t-RNA anti-RNase (40 U/ μl), and 10 μl cocaine (10 mM). The percentage of binding of the (^{32}P) RNA aptamers to the

filters (background binding) is determined in the absence of receptor protein.

4. Incubate reactions for 40 min at room temperature.
5. In the meantime, mount the glass fiber filters on top of the filter sheet in the dot-blot filtration unit (Schleicher and Schuell). For duplicate determinations, two samples each of 45 μ l solution are spotted on separate filters.
6. Gentle suction is applied to the filtration unit. The filters are washed with 200 μ l incubation buffer.
7. The filters are removed and transferred into 5 ml scintillation fluid.
8. The filter-bound radioactivity is measured by scintillation counting.

3.10. Determination of the Binding Affinities Using IC_{50} Determinations

The IC_{50} value is the concentration at which a competitor (cocaine) displaces 50% of the radioactive ligand (32 P-RNA) from the nAChR. On the assumption that cocaine binds with an apparent dissociation constant (K_d) of 50 μ M to the membrane-bound nAChR (42), the K_i of the RNA:nAChR complex is calculated from this data, using the equation of Cheng and Prusoff (43) where $K_i = IC_{50} / (1 + [^{32}\text{P} - \text{RNA}] / K_d)$. (32 P-RNA) is the radioligand concentration used in the experiment.

1. For IC_{50} determinations, incubate 5×10^5 cpm (32 P)-ATP-labeled RNA in the presence of 20 nM unlabeled RNA with constant concentrations of nAChR protein.
2. Increase cocaine concentration from 0 to 10 μ M and separate the reaction mixtures as described in Subheading 3.9.

3.11. Identification and Characterization of Individual Aptamers

1. For cloning and sequencing of individual RNA aptamers, nine SELEX cycles are necessary to obtain high-affinity RNA ligands for the nAChR.
2. Reverse transcribe the final RNA pool, and amplify and gel-purify the resulting cDNA (see Subheading 3.7).
3. Cut the constant regions using the restriction enzymes EcoRI and HindIII.
4. Purify the 69-bp reaction product on a gel made of low-melting agarose (Sea Plaque GTG).
5. Cut pGEM-3Z vector (Promega) using EcoRI and HindIII and gel-purify the linearized vector.
6. Ligate the purified DNA into the linearized pGEM-3Z vector.
7. Transform *E. coli* JM109 or DH5 α cells with the vector according to the instructions of Sambrook and Russell (40).
8. After streaking the transformed cells on ampicillin-selective LB-agar plates, grow the cells overnight.

9. Isolate individual white colonies, each containing one aptamer sequence.
10. Grow the isolated cells to the desired optical density (1.8–2.0 O.D. at 600 nm).
11. Purify the plasmids using the Wizard DNA Miniprep kit (Promega), and sequence the inserts according to the protocol supplied with the Big Dye sequencing kit (Applied Biosystems).
12. Then identify the consensus sequences and predict the aptamer secondary structure. The previous random sequences of the cloned RNA molecules are aligned and compared for consensus motifs found in almost all RNA molecules by simply aligning the sequences in a word processing program and visualizing sequence similarities by eye or the “find” function of the program, or by using a sequence alignment computer program. A thorough analysis of identifying aptamer consensus motifs was published (44). The corresponding aptamer secondary structures are predicted using the multifold computer program (41).
13. Amplify plasmid inserts coding for individual RNA aptamers by PCR using the primers P-40 and P-22pGEM and purify the PCR products on a low-melting agarose gel.
14. The purified DNAs are used as templates for in vitro transcription reactions. Forward primer (P-40, same sequence as primer used during SELEX process) and reverse primer (p-22 pGEM).

3.12. Screening for Binding Affinity and Biological Activity

1. Screening for binding affinity: Sequences containing consensus regions are in vitro transcribed in the presence of (^{32}P) UTP and tested for their affinity towards membrane preparations from *Torpedo* organs containing nAChRs (see Subheading 3.9).
2. Screening for biological activity: The biological activity of the selected RNA aptamers are determined in vitro, as to whether they inhibit the nAChR function as cocaine does or whether they compete with cocaine but do not have any biological activity by themselves and, therefore, protect the receptor against inhibition by cocaine (25).
3. For the electrophysiological assay, BC₃H1 cells that express the muscle-type nAChR are plated in cell culture dishes.
4. A patch-clamp (recording) pipette is attached to an individual cell and a gigaohm seal is formed between the cell and the patch-clamp pipette by applying suction through the pipette.
5. The whole-cell configuration is achieved by breaking the cell membrane; the buffer in the recording pipette is now in free exchange with the cytosol of the cells.

6. The flow of ions across the plasma membrane is recorded as current. An extensive description of the patch-clamp technique is given in ref. (30).
7. Currents induced by carbamoylcholine in the absence or the presence of cocaine and/or RNA aptamers are recorded by using the whole-cell recording technique (31) and the cell-flow method for rapid solution exchange and correction of the current for desensitization that occurs during the rising phase of the current (32). The recordings are made with a cell bathed in incubation buffer at a holding potential of -60 mV, pH 7.4, and 22°C . The recorded currents are amplified by using a patch-clamp amplifier and low-pass Bessel filtered at 1–2 kHz. The filtered signal is digitized on a 1322A-Digidata interface controlled by the Axon pCLAMP software. The recorded currents are corrected for receptor desensitization as has been described (32).
8. In vitro transcribe the large amounts of aptamers needed for electrophysiological experiments using the Megascript kit (Ambion).
9. Prepare BC₃H1 cells by plating them on 35-mm cell culture dishes and culture them for 14 days in subconfluent conditions in DMEM medium (high glucose) containing 1% FBS at 37°C and 7.5% CO₂.
10. Use filtered incubation buffer as the extracellular buffer and pipette solution (intracellular buffer), to fill the recording pipette.
11. Pull and fire polish patch pipettes when filled with buffer solution show a resistance of 3–5 M Ω and a series resistance of 5–6 M Ω .
12. Use the cell-flow technique (32) to determine the inhibition of the receptor. In these experiments, cells containing the nAChR are used. The maximum current amplitude, a measure of the concentration of open receptor-channels, is determined. The experiments are done in the presence of 100 μM carbamoylcholine (a stable analog of acetylcholine) and in the absence and presence of 150 μM cocaine.
13. Repeat the experiment with 100 μM carbamoylcholine in the presence of increasing concentrations of the RNA aptamer to be tested, in order to determine if the RNA aptamer inhibits receptor function. If an RNA aptamer does not inhibit receptor function, experiments are performed in the presence of a constant concentration of carbamoylcholine and cocaine with increasing concentrations of the RNA aptamer to determine whether the aptamer alleviates inhibition (25).

4. Notes

1. For precipitation of DNA and RNA, add 10% of the volume sodium acetate, pH 7 or 5.2, respectively. Add four parts of ice-cold 98% ethanol and mix well. Add 1 μ l of linear acrylamide (3%). Incubate for 2 h at -20°C and centrifuge at $12,000 \times g$ for 30 min in a tabletop centrifuge. Carefully remove the supernatant—the DNA or RNA should be visible against the light as one or several small shiny specks, usually attached to the tube, but they may be loose—and wash the pellet with 200 μ l 80% ethanol. Remove the supernatant, and dry the DNA or RNA (3 min in a speed vacuum concentrator) until all visible liquid has gone, but avoid overdrying of the pellet because the DNA or RNA will then be difficult to redissolve. Dissolve the DNA in 100 μ l dd H_2O .
2. In order to avoid the selection and amplification of RNA molecules that do not bind to the target site, two assays for in vitro selection are both employed after SELEX cycle 3. In addition a negative preselection step can be used at which unspecific binders to nitrocellulose (used for separation of receptor-bound from unbound RNA molecules) are discarded (see Subheading 3.7).
3. Too many PCR cycles will result in the occurrence of multimers of denatured DNA molecules that will migrate in the high molecular weight range in an analyzing PAGE. These sequences cannot be renatured to double-stranded DNA molecules and are lost, as they cannot be in vitro transcribed.
4. For denaturing PAGE for RNA: Prepare a 40% stock solution weigh out 38.5 g acrylamide ultrapure (pay attention is carcinogenic) and 25 g N,N' methylenebisacrylamide ultrapure. Dissolve in 100 ml DEPC-treated H_2O , microwave the solution for 40s, filter it and warrant protection from light. Prepare 10 \times TBE and 10 M urea solution in DEPC-treated H_2O (8 ml 40% acrylamide solution, 4 ml 10 \times TBE buffer, 28 ml dd H_2O). Fill between the glass plates and start polymerization. Add 30 μ l N,N,N',N' -tetramethyl-ethylenediamine (TEMED) and 300 μ l ammonium persulfate (APS) (10% solution).

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