Chapter 2

SELEX of Cell-Specific RNA Aptamers

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Abstract

This chapter focuses on the selection of RNA aptamers, which bind to specific cell surface components and thus can be internalized receptor mediated. Such aptamers discriminate between different tissues, e.g., detect malignant cells, and target them or induce apoptosis through drug internalization. However, before starting the selection process the choice of an ideal target can be challenging. To give an example for the selection of cell specific aptamers, we here used the interleukin-6 receptor (IL-6R) as a target, which is presented on hepatocytes, neutrophils, monocytes, and macrophages.

Key words Interleukin-6 receptor, SELEX, Internalization, Delivery

1 Introduction

Cell surfaces exhibit a high amount of diverse lipids, proteins, and carbohydrates, whereas their composition differs tremendously between various cell types. Therefore aptamers can be selected for a variety of targets like growth factors, adhesion molecules, and cell surface receptors which are exclusively presented on special cell types [1]. Such aptamers can differentiate between cell types, for example healthy and tumor cells or even different types of cancer cells [2–5]. Therefore cell-specific aptamers have a high potential for diagnosis and as therapeutics.

By targeting a cell surface molecule that underlies natural internalization processes, an aptamer can act as a molecular carrier to convey fluorophores, chemotherapeutics, or other agents into cells [3, 6–9]. A prerequisite is that binding of the aptamer (including its cargo) does not interfere with the natural transport mechanism of the receptor.

In some cases, aptamer binding can interfere with receptor-ligand interaction or the receptor recycling processes, thus inhibiting the binding of natural ligands and/or altering the subsequent signal transduction [10]. However, recycling processes of most cell surface lipids, receptors, or tumor markers are still not completely

understood. Though there are some reports on internalized cell specific aptamers, the localization of both aptamer and delivered cargo cannot be predicted and determined easily and differs depending on the respective receptor and cargo.

One example of such an aptamer represents PSM-A10, an RNA aptamer, which inhibits the prostate-specific membrane antigen (PSMA) with a K_i of 11.9 nM. PSM-A10 is able to bind LNCaP human prostate cancer cells presenting PSMA, but not PSMA-devoid PC-3 human prostate cancer cells [4]. It can be used for the localized delivery of siRNA molecules, polymer nanoparticles, or toxins like gelonin [6–8].

Another example is an RNA aptamer named AIR-3 as well as its truncated form AIR-3A, which interact with high affinity (K_d = 20 nM) and specificity with the human interleukin-6 receptor (IL-6R) [11]. AIR-3A is also able to deliver therapeutic agents, such as chlorin e6 (ce6), into its target cell. This enables a specific ce6-mediated photodynamic therapy [12]. AIR-3 even might be used for targeted chemotherapy when intrinsically comprising the cytostaticum 5-fluoro-2′-deoxy-uridine [13].

The selection of aptamers consists of three main steps: The first step is the incubation of an RNA library with the target molecule. This library consists of around 10^{14} different sequences, which share a randomized region of 30–50 nucleotides (N30–50). This region is flanked by two constant regions for T7-transcription and RT-PCR.

After incubation, bound and unbound oligonucleotides are separated. In a common in vitro selection procedure, the target molecule is immobilized on a column or beads, while other protocols utilize gel electrophoresis or nitrocellulose membrane filtration [14, 15]. In the last step of each SELEX cycle, bound molecules are eluted, amplified via RT-PCR, and then transcribed to generate an enriched pool of RNA oligonucleotide species for the next selection round. This iterative process is repeated 5–15 times, followed by cloning and sequencing of the dsDNA library [16, 17].

Obtained oligonucleotides can be characterized for their binding abilities and their specificity either by protein-RNA interaction assays like filter retention, electric mobility shift, and plasmon resonance assays or directly on cells using flow cytometry or confocal microscopy. Subsequently to the SELEX procedure, the affinity of the aptamers to cells has to be determined. Therefore, the aptamer is fluorescently labelled. Adequate control cells omitting the target protein are required to confirm specificity of the aptamers and allow characterizations (e.g., toxicity, inhibiting or activating events, aptamer internalization).

The interleukin-6 receptor, used as an aptamer target by our group, interacts with its cytokine interleukin-6 (IL-6) and two glycoproteins 130 (gp130). The resulting gp130 dimerization

initiates an intracellular signal transduction, in which event the expression of various genes is activated [18, 19]. We used Ba/F3/ gp130/IL6R/TNF cells to analyze the influence of the aptamer on signal transduction and examined receptor mediated endocytosis. As a control cell line Ba/F3/gp130/TNF cells without IL-6R were used.

Generally, before starting a SELEX there are some aspects, which have to be taken into account. In most cases, it is indispensable to use a soluble form of the target, often corresponding to the extracellular domain of a cell surface protein if that has been chosen. Nevertheless it needs to be ensured, that the protein is correctly folded and fully functional. If the cell surface protein of choice cannot be produced in its soluble form, cell-SELEX [20–22] would be a favorable alternative. For a successful SELEX experiment, it is desirable to have as much information as possible about the structure as well as the physical and chemical properties of the target protein. This is required to assess ideal buffer conditions, immobilization of the target and an appropriate pre- or counterselection molecule to enhance the selectivity. Some chosen targets might be highly glycosylated or some just might be difficult to purify, rendering selection more challenging.

If the selected RNA aptamer is planned to be used in vivo, it might be necessary or at least advantageous to enhance its stability. Post-selective modifications can interfere with aptamer folding and target interaction, it is therefore recommended to use a stabilized RNA library (e.g., with 2′-*O*-methyl or 2′-fluoro modifications) directly in the SELEX process [21, 23–25]. For certain applications, DNA aptamers might be preferential [26].

2   Materials

Prepare all solutions RNase-free (*see* **Note 1**).

2.1 Preparation of RNA Library and In Vitro T7 Transcription

1. 100 μM single strand DNA library: 5′-AATGCTAATACGACT CACTATAGG AAGAAAGAGGTCTGAGACATTCT–N50– CTTCTGGAGTTGACGTTGCTT-3′ (*see* **Note 2**).

2. 100 μM RT primer: 5′-AAGCAACGTCAACTCCAGAAG-3′

3. Klenow Fragment (5 U/μL), 10× Klenow Fragment buffer: 500 mM Tris-HCl (pH 8.0 at 25 °C), 50 mM $MgCl_2$, 10 mM DTT.

4. 10× hybridization buffer: 200 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM EDTA.

5. dNTP mix: 25 mM ATP, 25 mM CTP, 25 mM GTP, 25 mM TTP.

6. 20 U/μL T7 RNA Polymerase (*see* **Note 3**).

7. 5× transcription buffer: 200 mM Tris-HCl, pH 7.9.

8. 100 mM $MgCl_2$.

9. NTP-mix A: 25 mM ATP, 25 mM CTP, 25 mM GTP, 25 mM UTP (*see* **Note 4**).

2.2 Biotinylation of Target Protein

1. 100 μg soluble IL-6R (sIL_6R) (Conaris).

2. 2 mg *Sulfo*-NHS-LC-Biotin.

3. Phosphate-buffered saline (PBS): 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 6.5 mM NaH_2PO_4, pH 7.4.

4. SELEX buffer: 3 mM $MgCl_2$ in PBS (*see* **Note 5**).

5. Slide-A-Lyzer® dialysis cassette (MWCO 10K; Thermo Fisher Scientific) (*see* **Note 6**).

6. Nitrocellulose membrane.

7. ExtrAvidin® Alkaline phosphatase.

8. Blocking buffer: 5 % (w/v) skim milk powder in PBS.

9. AB buffer: 2.5 % (w/v) skim milk powder in PBS.

10. Reaction buffer: 100 mM Tris-HCl, pH 9.5, 4 mM $MgCl_2$.

11. AP solution: 0.01 % (w/v) 5-bromo-4-chloro-3-indoxyl phosphate, 0.002 % (w/v) nitro blue tetrazolium chloride in reaction buffer.

2.3 Immobilization on Streptavidin-Coated Dynabeads

1. Dynabeads® M-280 Streptavidin (Invitrogen).

2. Magna-Sep™ Magnetic Particle Separator.

3. SELEX buffer: 3 mM $MgCl_2$ in PBS.

4. BSA solution: 20 μg/μL in water.

5. Coupling buffer A: 1 μg BSA/μL, 1× PBS, pH 7.4.

6. Coupling buffer B: 1 μg BSA/μL, 1.25× PBS, pH 7.4.

2.4 Reverse Transcription-Polymerase Chain Reaction

1. 5 U/μL FIREPol® DNA Polymerase (Solis BioDyne).

2. 10× PCR reaction buffer B: 0.8 M Tris-HCl, 0.2 M $(NH_4)_2SO_4$, 0.2 % w/v Tween-20 (Solis BioDyne).

3. 15 U/μL SuperScript™ III Reverse Transkriptase.

4. 5× cDNA synthesis buffer: 250 mM Tris-acetate, pH 8.4, 375 mM potassium acetate, 40 mM magnesium acetate.

5. 25 mM $MgCl_2$.

6. 100 mM DTT.

7. dNTPmix: 25 mM ATP, 25 mM CTP, 25 mM GTP, 25 mM TTP.

8. 100 μM RT primer: 5′-AAGCAACGTCAACTCCAGAAG-3′.

9. 100 μM T7 primer: 5′-<u>AATGCTAATACGACTCACTATAG</u> GAAGAAAGAGGTC TGAGACATT-3′.

2.5 Cloning	1. TOPO TA Cloning Kit (pCR2.1).
2.6 Filter Retention Assay	1. [α-^{32}P]-ATP (3000 Ci/mmol, 10 mCi/mL, Hartmann Analytic).
	2. NTP-mix B: 25 mM ATP, 25 mM CTP, 25 mM GTP, 25 mM UTP.
	3. SELEX buffer: 3 mM MgCl$_2$ in PBS.
	4. Aminohexanoic acid buffer: 20 % (v/v) Methanol; 40 mM 6-aminohexanoic acid.
	5. Filtering paper (e.g., Rotilabo®-Blottingpaper, Carl Roth).
	6. 500 μM sIL-6R (Conaris).
	7. Nitrocellulose membrane (0.45 μm).
	8. Minifold® I dot-blot-system (e.g., Schleicher & Schuell).
2.7 Flow Cytometry	1. 10 μM fluorescently labeled RNA (*see* **Note 7**).
	2. Mouse anti-human Interleukin-6 receptor antibody (Bender MedSystems) (*see* **Note 7**).
	3. Mouse anti-human gp130 antibody (R&D Systems) (*see* **Note 8**).
	4. Goat anti-mouse Ig–APC (BD Pharmingen).
	5. Ba/F3/gp130/IL6R/TNF cells.
	6. Ba/F3/gp130/TNF cells.
	7. SELEX buffer: 3 mM MgCl$_2$ in PBS.
	8. FACSCalibur flow cytometer (BD Bioscience).

3 Methods

3.1 Preparation of RNA Library	1. Heat 2.5 μL ssDNA library (100 μM) with 5 μL RT primer (100 μM), 5 μL hybridization buffer (10×), and 37.5 μL water to 95 °C for 5 min and cool down slowly to room temperature afterwards.
	2. Mix 50 μL hybridization product with 1 μL Klenow Fragment (10 U/μL), 10 μL Klenow Fragment buffer, 2 μL dNTPs (25 mM), and 37 μL water and incubate for 1 h at 37 °C. Then inactivate Klenow Fragment by heating to 75 °C for 10 min.
	3. For in vitro transcription, mix 20 μL double-strand DNA library with 20 μL transcription buffer (5×), 15 μL MgCl$_2$ (100 mM), 10 μL NTP-Mix (25 mM), 1 μL T7 RNA Polymerase (25 U/μL), and 34 μL water and incubate for 30 min at 37 °C.
3.2 Biotinylation of Target Protein	1. Dissolve 2 mg Sulfo-NHS-LC-Biotin in 360 μL water (yielding a 10 mM solution, which needs to be diluted to 1 mM) (*see* **Note 9**).

2. Mix 100 µL sIL-6R (1.15 µg/µL) with 8.8 µL freshly prepared Sulfo-NHS-LC-Biotin reagent (1 mM) and incubate on ice for 15 min. Afterwards incubate for additional 15 min at room temperature (*see* **Note 10** and **11**).

3. Remove excess of non-reacted and hydrolyzed biotin reagent by dialysis with Slide-A-Lyzer® dialysis cassette. Therefore, fill mixture into the dialysis cassette and dialyze in 500 mL stirring SELEX buffer for 1 h at 4 °C. Then exchange the buffer and dialyze the mixture overnight.

4. Verify biotinylation by dot blot analysis. Therefore, place 3 µL of biotinylated protein on a nitrocellulose membrane and 3 µL non-biotinylated protein next to it. Let drops dry up.

5. Place the membrane in 25 mL blocking buffer for 1 h at room temperature and swing gently. Next, wash membrane briefly in 25 mL PBS, place the membrane in 25 mL AB buffer with 10 µL ExtrAvidin® Alkaline phosphatase for 1 h at room temperature, and swing gently again.

6. Wash the membrane twice in 25 mL PBS and once in 25 mL reaction buffer for 5 min at room temperature, respectively.

7. Add 25 mL reaction buffer to the membrane including 12.5 µL AP solution and incubate until color reaction significantly appears. To stop reaction transfer the membrane into water.

3.3 Immobilization on Streptavidin-Coated Dynabeads

1. Transfer 500 µL Dynabeads® into a reaction tube and put it into the magnetic particle separator for magnetic separation (*see* **Note 10**). Discard supernatant, take reaction tube out of separator, and resolve the beads in 1 mL coupling buffer A (*see* **Note 12**). Repeat this washing step four times.

2. Add 100 µg biotinylated protein to the beads and incubate for 15 min at 4 °C. Wash the beads five times with 500 µL coupling buffer A and then twice with 500 µL coupling buffer B.

3. Store the beads in 1.5 mL coupling buffer B at 4 °C.

3.4 SELEX

1. For the initial round of selection, mix 500 pmol of the RNA library with 100 pmol target protein immobilized on magnetic beads in SELEX buffer and incubate for 30 min at 37 °C. Discard unbound RNA by magnetic separation (*see* Subheading 3.3) and wash beads with 200 µL SELEX buffer (*see* **Note 13**).

2. Elute bound RNA by resuspending magnetic beads in 50 µL water and heat for 3 min at 80 °C. Then separate RNA containing water from magnetic beads by magnetic separation and transfer supernatant to a new reaction tube for reverse transcription-polymerase chain reaction (RT-PCR).

3. For RT-PCR mix the following components: 50 µL eluate from **step 2**, 10 µL 10× PCR reaction buffer B, 4 µL 5× cDNA

synthesis buffer, 6 μL 25 mM $MgCl_2$, 1.2 μL dNTPmix, 1 μL RT primer, 1 μL T7 primer, 2 μL DTT, and 22.8 μL water. Incubate samples at 65 °C for 5 min and cool down to room temperature afterwards.

4. Add 1 μL SuperScript™ III Reverse Transcriptase and 1 μL FIREPol® DNA polymerase and incubate reaction mix for 10 min at 54 °C for reverse transcription.

5. Use reaction mixture from **step 4** instantly for PCR amplification comprising following steps: denaturing for 30 s at 95 °C, annealing for 30 s at 60 °C and elongation for 30 s at 72 °C and appropriate number of PCR cycles (*see* **Note 14**). Verify PCR product by performing standard polyacrylamide gel electrophoresis.

6. Transcribe resulting double stranded DNA (dsDNA) of **step 5** into RNA. Therefore, mix 10 μL dsDNA with 20 μL 5× transcription buffer, 15 μL $MgCl_2$, 10 μL NTP-mix A, 1 μL T7 RNA polymerase and 44 μL water and incubate for 30 min at 37 °C.

7. Verify RNA by performing polyacrylamide gel electrophoresis.

8. Start new selection round with 20 μL obtained RNA (*see* **Note 15**).

9. To investigate individual clones, clone dsDNA library via TOPO TA cloning (*see* **Note 16**) and perform sequence analysis.

3.5 Filter Retention Assay

1. Perform T7 transcription to radioactively label RNA. Therefore, mix 10 μL dsDNA with 20 μL 5× transcription buffer, 15 μL $MgCl_2$, 10 μL NTP mix B, 1 μL [α-^{32}P]-ATP, and 1 μL T7 RNA polymerase with 43 μL water and incubate overnight at 37 °C.

2. Isolate RNA product by gel purification and dissolve RNA in 20 μL of water.

3. Mix 1 μL of radioactively labeled RNA (<1 nM) with 199 μL SELEX buffer.

4. Dilute target protein in SELEX buffer to a concentration of 5 μM in a total volume of 12 μL (*see* **Note 17**). For serial protein dilution mix 6 μL 5 μM protein with 6 μL SELEX buffer and continue to dilute the protein four times.

5. Start binding assay: mix 5 μL of each protein dilution with 20 μL RNA (**step 3**) and incubate for 30 min at 22 °C. As negative control, dilute 20 μL RNA with 5 μL SELEX buffer (*see* **Note 18**).

6. Cut one filter paper and nitrocellulose membrane to the size of the dot blot apparatus. Equilibrate nitrocellulose membrane in aminohexanoic acid buffer for 30 min at room temperature. Afterwards, equilibrate both nitrocellulose membrane and filter paper in SELEX buffer for at least 5 min.

7. Assemble dot blot apparatus. Therefore, place filter paper beneath the nitrocellulose membrane inside dot blot apparatus.

8. Apply vacuum on dot blot apparatus and wash each well twice with 200 µL SELEX buffer.

9. Transfer all samples into the dot blot apparatus in respective wells and let the samples completely pass through the membrane (*see* **Note 19**).

10. Wash each well twice with 200 µL SELEX buffer and let buffer completely pass through the membrane. Then take out the nitrocellulose membrane and let it dry.

11. Prepare 100 % controls. Mix 5.4 µL SELEX buffer with 0.6 µL radioactively labeled RNA. Apply twice 2 µL to the membrane and let it dry.

12. Transfer membrane into a plastic bag and expose properly until analysis.

13. For analysis use a phosphor imager.

14. Quantify intensities of the dots and calculate dissociation constant (Fig. 1).
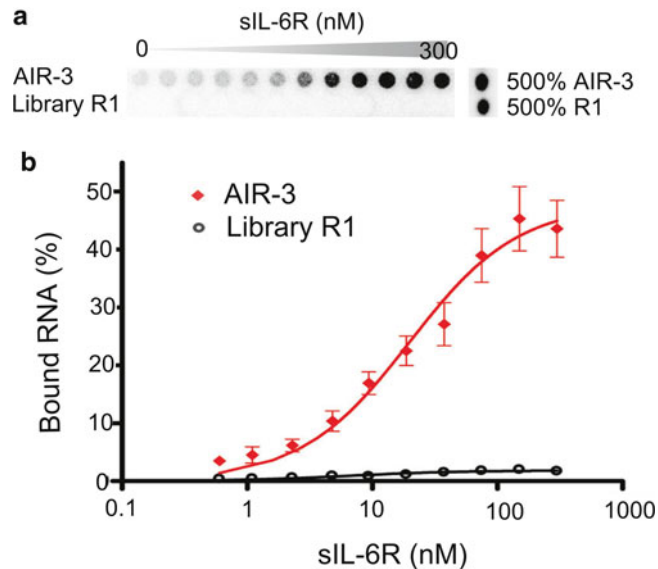

Fig. 1 (**a**) Filter retention assay. Constant amounts (<1 nM) of ^{32}P-radioactively labeled aptamer AIR-3 or RNA starting library R1 were incubated with increasing amounts of sIL-6R (0–300 nM). Protein-bound RNA could be visualized by autoradiography. To quantify the amount of RNA a 500 or 100 % control is used. (**b**) To calculate dissociation constants, fractions of bound RNA molecules (AIR-3A, *red*; library, *black*) were plotted against the concentrations of sIL-6R (logarithmic scale). Data points represent mean values of ten independent measurements (further details *see* [11])
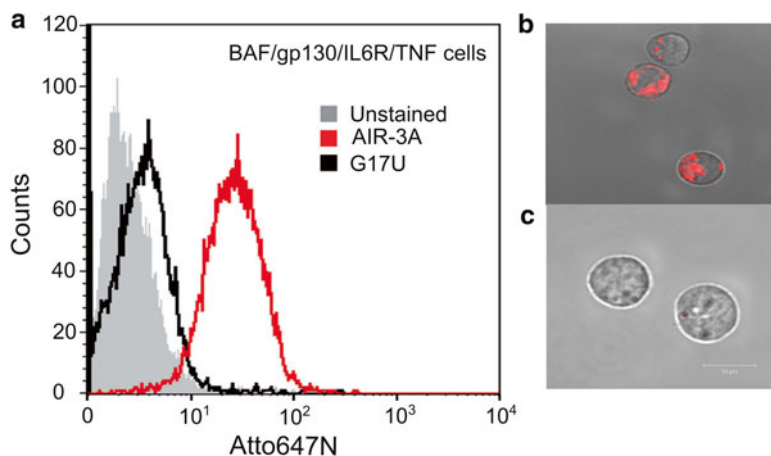
Fig. 2 (**a**) Flow cytometry analysis. Upon binding of Atto-647 N-labeled Aptamer AIR-3A (*red*) to BAF/gp130/IL6R/TNF cells presenting the human IL-6 receptor, fluorescence shifts to higher values. Cells incubated with the variant G17U (Atto647N-labeled control, *black*) resulted in a smaller shift compared to AIR-3A. Laser scanning microscopic image of AIR-3A (**b**) or its variant G17U (**c**) with BAF/gp130/IL6R/TNF cells. The aptamer (**b**) is internalized into these cells (further details *see* [11])

3.6 Flow Cytometry

Perform all steps at 4 °C and use SELEX buffer pre-cooled to 4 °C (*see* **Note 20**).

1. Count cells and use 1×10^5 cells for each sample.

2. Wash cells twice. Therefore, pellet cells for 3 min at $800 \times g$ and resuspend with 350 µL SELEX buffer (*see* **Note 21** and **22**).

   (a) Control cells are not further treated

   (b) Aptamer binding: Add 1 µL fluorescently labeled RNA aptamer (10 pmol) to the cells and incubate for 30 min on ice.

   (c) IL-6R and gp130 verification: Add either 1 µL IL-6R antibody or gp130 antibody to the cells and incubate for 30 min on ice. Then wash cells once with SELEX buffer and add 1 µL anti-mouse Ig–APC antibody to the cells and incubate for another 30 min on ice.

3. Wash cells once with 350 µL SELEX buffer.

4. Determine fluorescence intensities by flow cytometry analysis and compare intensities (Fig. 2).

4 Notes

1. Aqueous solutions can be treated with diethylpyrocarbonate (DEPC) to inactivate RNases due to acetylation of histidine, lysine, cysteine and tyrosine residues. Therefore solution is

treated with 0.1 % (v/v) DEPC and stirred overnight. Autoclaving afterwards leads to destruction of DEPC. Tris buffered solutions should not be treated with DEPC as it inactivates DEPC. Alternatively, RNase-free water derived from special filtration plants can be used.

2. Given are sequences of a library used for the selection of aptamers binding to the human interleukin-6 receptor [11]. Randomized region can be optimized regarding length and given secondary structure (e.g., inserting a stem loop region). Optimal lengths of randomized region are between 20 and 50 nucleotides.

3. Instead of wild type RNA polymerase, variants (e.g., RNAP Y639F or R425C) can be used which are able to incorporate modified nucleotides.

4. Unmodified standard nucleotides can be replaced by modified nucleotides (e.g., 2′-fluoronucleotides, *O*-methyl nucleotides), which require the usage of specialized RNA polymerases. Mostly the reaction time needs to be increased to 4 h instead of 30 min.

5. Alternatively, other dialysis cassettes, membranes, tubes, and suchlike can be used.

6. If the target protein exhibits a very low isoelectric point the use of positive ions, e.g., calcium ions are recommended.

7. Fluorescently labeled RNA can be purchased or labeled by inserting fluorescing nucleotide analogs during T7 transcription (e.g., guanosine-5′-*O*-(3-thiotriphosphate)).

8. Choose antibodies which are applicable for FACS measurements.

9. There are several types of magnetic beads to choose from. In the given example streptavidin coated beads were used, but also the chemical coupling to carboxyl beads is possible.

10. Prepare *Sulfo*-NHS-LC-Biotin reagent immediately before usage.

11. *Sulfo*-NHS-LC-Biotin reagent should be used at threefold molar excess to the protein.

12. Dynabeads® are paramagnetic polystyrene particles that can be separated by a magnet. Placing a reaction tube which contains Dynabeads® into the Magnetic Particle Separator always leads directly to magnetic separation. Successful separation can be observed when solution cleared up.

13. Prevent the magnetic beads to become dry.

14. In the course of selection, the number of washing steps is increased to favor the selection of high affinity nucleic acids. So start with one washing step and add one step in each selection round.

15. The optimal number of PCR cycles lies between 4 and 20. To prevent formation of PCR side products emulsion PCR (e.g.,

Micellula DNA Emulsion & Purification Kit by EUR$_X$) can be performed alternatively [27].

16. Stop SELEX procedure after 10–15 rounds. The number of selection rounds depends on the affinity of the nucleic acid pool to the target. If the whole library is analyzed by next generation sequencing less round are required.

17. To increase the enrichment of nucleic acids which are only binding to the target molecule and not to Dynabeads® or Streptavidin, it is common to do a preSELEX prior to each SELEX round. Therefore use Dynabeads® with only biotin and incubate them at 37 °C for 30 min with the prior round first. Then use the supernatant as described above in the actual SELEX round. In this step it is also possible to use Dynabeads® coupled with non-target molecules to exclude those nucleic acids which are not binding specifically.

18. Using a Taq polymerase for polymerase chain reaction leads to adenine overhangs at the 3′-end of the PCR product. These overhangs are required for successful TA cloning. Alternatively to the mentioned TOPO TA cloning kit, a vector can be used which exhibits 5′-thymine overhangs after restriction (e.g., endoribonuclease XcmI), or standard cloning can be performed at restriction sites which were added during polymerase chain reaction or presented within the primer sites.

19. 12 μL 5 μM protein solution is needed to perform one binding assay with one sample of RNA. If several aptamers are analyzed simultaneously, protein amount has to be increased proportionally.

20. End concentrations of protein are 1000, 0.500, 0.250, 0.125, 0.063, and 0.031 μM.

21. Avoid touching the nitrocellulose membrane with the tip. The membrane can be damaged and distort results.

22. Resuspend cells gently, prevent formation of foam, and carry out all steps quickly without delay to prevent cell death.

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