Chapter 2

In Vitro Selection of ssDNA Aptamers Using Biotinylated Target Proteins

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Abstract

Aptamers are single-stranded nucleic acids that bind specifically to a target molecule and thus often inhibit target-associated biological functions. Aptamers have been described for a series of target molecules including peptides, proteins, and even living cells. Besides RNA and 2′-modified RNA molecules also ssDNA molecules can be subjected to in vitro selection protocols aiming at the enrichment of ssDNA aptamers. ssDNA aptamers can be selected using the SELEX procedure (systematic enrichment of ligands by exponential amplification) from libraries of randomized single-stranded DNA with a diversity of up to 10^{16} different molecules. In repetitive selection cycles, the library is incubated with the target of choice and separation of non-binding sequences from bound sequences is achieved by distinct separation methods. The bound molecules are specifically eluted and amplified, thus representing the starting library for the next cycle. Thereby, an enriched population of aptamers is evolved. Here we describe a generalized in vitro selection experiment aiming at the enrichment of ssDNA aptamers using biotinylated target molecules. This procedure allows the application of streptavidin–biotin chemistry to separate bound from unbound DNA species during the selection process.

Key words: SELEX, aptamers, ssDNA, in vitro selection, biotin, streptavidin.

1. Introduction

Nucleic acids are able to fold into well-defined three dimensional structures *(1)* that enable them to interact with a great number of different targets. This ability has been exploited in the SELEX process (systematic evolution of ligands by exponential enrichment), which allows the selection of single-stranded nucleic acids that bind specifically and with high affinity to their cognate target molecules *(2, 3)*. These nucleic acids are termed as aptamers. In several cases, the interaction of the aptamer with its cognate target

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molecule is accompanied by inhibition of target-associated biological functions *(4–6)*. The SELEX method, originally described for RNA aptamers, has consequently been adapted for ssDNA yielding DNA-based aptamers. In this manner aptamers against numerous targets including proteins, small molecules, viruses, and whole cells *(7–9)* have been identified and ssDNA aptamers that target thrombin have been developed into clinical trials as *anti*-thrombotic agents *(10, 11)*. During the SELEX process a starting library *(12)* of ssDNA molecules with a random region flanked by defined primer binding sites is incubated with the target molecule, either in solution or coupled to a solid matrix. In the following, non-binding sequences are removed by washing steps and the bound species are eluted and amplified by PCR. After denaturation of the dsDNA, the counter-strands will be removed to generate an enriched library of ssDNA. This library represents the starting point for the next selection cycle. Radioactive labelling of the DNA allows the detection of binding sequences in a filter-retention analysis assay. The coupling of target protein to solid supports can be achieved either by non-covalent or covalent attachment of the proteins to sepharose-based matrices, such as CNBr-activated sepharose or thiopropyl sepharose. Here we describe an alternative approach that makes use of biotinylation of a target protein and its subsequent coupling to magnetic beads coated with streptavidin. These beads can be implemented in selection schemes for the successful enrichment of aptamers. This selection scheme can be generalized and applied to a variety of target proteins amenable to the NHS-chemistry-based biotin modification.

2. Materials

2.1. Biotinylation

1. 10x phosphate-buffered saline (PBS): 1.37 M NaCl, 27 mM KCl, 65 mM Na_2HPO_4 and 14.7 mM NaH_2PO_4. Adjust to pH 7.4 with HCl and NaOH. Store at room temperature (*see* **Note 1**).
2. Sulfo-NHS-LC-Biotin (Pierce). Prepare a fresh 1.8 mM solution in H_2O as required.
3. Bio-Spin Chromatography Columns P6 (Bio-Rad).

2.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Separating buffer: 1.5 M Tris–HCl, pH 8.8. Store at room temperature.
2. Stacking buffer: 1 M Tris–HCl, pH 6.8. Store at room temperature.

3. 10% SDS solution. Store at room temperature.
4. 30% bis-acrylamide (Roth). Store at 4°C. Bis-acrylamide is a neurotoxin. Be careful and avoid direct contact.
5. 10% ammoniumperoxodisulphate solution (APS). Store at 4°C.
6. N,N,N',N'-Tetramethylethylendiamin (TEMED). Store at 4°C.
7. Isopropanol.
8. Running buffer: Prepare 10x glycine electrophoresis buffer: 250 mM Tris–HCl, pH 8.9, 2 M glycine, 1% SDS (w/v). Dilute 1:10 with water prior use.
9. Prestained molecular weight markers.
10. SDS-PAGE loading buffer: prepare 8 ml 4x buffer by mixing: 4.3 ml water, 0.5 ml stacking buffer, 0.8 ml glycerol, 1.6 ml 10% SDS solution, 0.4 ml 2-mercaptoethanol, a spatula tip bromophenol blue. Store at –20°C.

2.3. Coomassie Staining

1. Staining solution: 375 mg Coomassie R-250 (Bio-Rad), 125 ml isopropanol, 50 ml acetic acid, 300 ml water.
2. Destaining solution: 30% (v/v) isopropanol, 10% (v/v) acetic acid.
3. Whatman-paper (Schleicher & Schuell).

2.4. Dot-Blotting

1. 10x PBS: 1.37 M NaCl, 27 mM KCl, 65 mM Na_2HPO_4 and 14.7 mM NaH_2PO_4. Adjust to pH 7.4 with HCl and NaOH. Dilute 1:10 with water prior use. Store at room temperature.
2. Blocking buffer: 1x PBS supplemented with 0.1 mg/ml BSA. Store at 4°C.
3. Fluorescently labeled antibody: Monoclonal *anti*-biotin (mouse IgG1 isotype) FITC-conjugate (Sigma).
4. Nitrocellulose transfer-membrane, pore size 0.45 µm (Protran, Whatman).

2.5. Preparation of the Matrix

2.5.1. Pre-selection Matrix

1. Dynabeads M-280 Streptavidin (Invitrogen, Dynal Biotech).
2. Washing buffer: 1x PBS supplemented with 1 mM $MgCl_2$.
3. 5x selection buffer: 685 mM NaCl, 13.5 mM KCl, 32.5 mM Na_2HPO_4, 9 mM NaH_2PO_4, 7.35 mM $MgCl_2$ and 0.5% (w/v) bovine serum albumin (BSA). Dilute to 1x buffer by mixing one part buffer with four parts water. Adjust to pH 7.4 with HCl and NaOH. Store at 4°C.
4. Magnetic particle concentrator rack (Invitrogen, Dynal Biotech).

2.5.2. Selection Matrix

1. Dynabeads M-280 Streptavidin (Invitrogen, Dynal Biotech).
2. Washing buffer: 1x PBS with 1 mM $MgCl_2$.

3. Selection buffer.
4. Magnetic particle concentrator rack (Invitrogen, Dynal Biotech).

2.6. Strand Displacement

1. 2x Bind and wash buffer (B & W buffer): 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 2 M NaCl. Dilute to 1x B & W buffer by mixing one part with same amount of water. Store at room temperature.
2. 0.15 M NaOH.
3. 0.3 M HCl.
4. Magnetic particle concentrator rack (Invitrogen, Dynal Biotech).
5. 5x selection buffer: 685 mM NaCl, 13.5 mM KCl, 32.5 mM Na_2HPO_4, 9 mM NaH_2PO_4, 7.35 mM $MgCl_2$ and 0.5% (w/v) bovine serum albumin (BSA).

2.7. SELEX

1. 5x selection buffer: 685 mM NaCl, 13.5 mM KCl, 32.5 mM Na_2HPO_4, 9 mM NaH_2PO_4, 7.35 mM $MgCl_2$ and 0.5% (w/v) bovine serum albumin (BSA).

2.7.1. First Cycle

2. DNA-library D1: 5′- GCC TGT TGT GAG CCT CCT AAC (N49) CAT GCT TAT TCT TGT CTC CC - 3′ (Metabion, *see* **Note 2**).

2.7.2. Strand Displacement

1. 2x binding and washing buffer (B & W buffer): 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 2 M NaCl. Store at room temperature.
2. 0.15 M NaOH.
3. 0.3 M HCl.
4. Magnetic particle concentrator rack (Invitrogen, Dynal Biotech).
5. 5x selection buffer: 685 mM NaCl, 13.5 mM KCl, 32.5 mM Na_2HPO_4, 9 mM NaH_2PO_4, 7.35 mM $MgCl_2$ and 0.5% (w/v) bovine serum albumin (BSA). Dilute to 1x buffer by mixing one part buffer with four parts water.

2.7.3. Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis

1. 10x $MgCl_2$-free Taq PCR-buffer (Promega).
2. 25 mM $MgCl_2$.
3. dNTPs (Sigma, Roche).
4. 100 μM Forward primer: 5′- GCC TGT TGT GAG CCT CCT AAC – 3’ (Metabion).
5. 100 μM Reverse primer with 5′ biotin tag: 5′(bio)- GGG AGA CAA GAA TAA GCATG - 3′ (Metabion).
6. Taq DNA-polymerase.

7. Agarose, electrophoresis grade (Invitrogen).
8. 10x TBE-buffer: 89 mM Tris–HCl (pH 8.0), 89 mM boric acid, 2 mM EDTA solution. Dilute 1:10 with water.
9. Ethidiumbromide (Roth). Ethidium bromide intercalates in DNA and is highly toxic.
10. Agarose gel-loading buffer: 50% glycerol, 50 mM Tris–HCl (pH 8.0), 50 mM EDTA (pH 8.0), optionally add one spatula tip of bromophenol blue and xylene cyanol.

2.7.4. SELEX Cycles 2-x

1. 5x selection buffer: 685 mM NaCl, 13.5 mM KCl, 32.5 mM Na_2HPO_4, 9 mM NaH_2PO_4, 7.35 mM $MgCl_2$ and 0.5% (w/v) bovine serum albumin (BSA).

2.8. 5′-End Labelling of ssDNA Molecules

1. T4 Polynucleotide-Kinase (New England Biolabs).
2. 10x PNK-Buffer (New England Biolabs).
3. γ-[^{32}P]-ATP (Perkin Elmer). This compound is radioactive and should be handled with great care and only with appropriate protection measures.

2.9. Polyacrylamide Gel Electrophoresis (PAGE)

1. Concentrated gel solution: 25% bis-acrylamide in 8.3 M urea. Bis-acrylamide is a neurotoxin. Be careful and avoid direct contact.
2. Thinner: 8.3 M urea.
3. Gel-buffer: 8.3 M urea in 10x TBE-buffer.
4. 10x TBE-buffer: 89 mM Tris–HCl (pH 8.0), 89 mM boric acid, 2 mM EDTA solution.
5. 10% ammoniumperoxodisulphate solution (APS). Store at 4°C.
6. TEMED. Store at 4°C.
7. PAGE-Gel loading buffer: 9 M urea, 50 mM EDTA (pH 8.0), a spatula tip of bromophenol blue and xylene cyanol. Store in aliquots at –20°C.

2.10. Filter-Retention Analysis

1. 5x selection buffer: 685 mM NaCl, 13.5 mM KCl, 32.5 mM Na_2HPO_4, 9 mM NaH_2PO_4, 7.35 mM $MgCl_2$ and 0.5% (w/v) bovine serum albumin (BSA). Dilute to 1x buffer by mixing one part buffer with four parts water.
2. 10 µg/µl tRNA from baker's yeast (Fluka).
3. Interaction assay mix: Mix 500 µl 5x selection buffer with 66 µl tRNA; add water to 1,900 µl. The mix is sufficient for 100 binding tests. Store at –20°C.
4. Washing buffer: 1x PBS with 1 mM $MgCl_2$.
5. 96 well Dot-blot unit (Minifold, Schleicher & Schuell).

6. Blotting paper (Whatman, Schleicher & Schuell).
7. Nitrocellulose transfer-membrane, pore size 0.45 µm (Protran, Whatman).

3. Methods

3.1. Biotinylation of Target Proteins

1. Mix 100 µg target protein with a threefold molar excess of sulfo-NHS-LC-biotin in a total volume of 100 µl 1x PBS (*see* **Note 3**).
2. Incubate on ice for 30 min and for further 15 min at room temperature.
3. Remove non-reacted sulfo-NHS-LC-biotin by gel filtration using P6 Spin-Columns. Safe a 5 µl aliquot of the reaction for SDS-PAGE and dot-blot analysis (*see* **Note 4**).

3.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Clean the glass plates with water and 70% ethanol before use.
2. Prepare a 12% separating gel by mixing 1,700 µl water, 1,250 µl separating buffer, 50 µl 10% SDS solution, 2,000 µl 30% bis-acrylamide, 25 µl 10% APS and 2.5 µl TEMED. Pour a 0.75 mm gel. Leave space for the stacking gel. Cover the gel with isopropanol and let polymerize for 30 min.
3. Pour off isopropanol and prepare a 4% stacking gel by mixing 1,220 µl water, 500 µl stacking buffer, 10 µl 10% SDS solution, 270 µl 30% bis-acrylamide, 10 µl 10% APS and 2.5 µl TEMED. Pour on top of the stacking gel and insert comb. Let polymerize for 30 min (*see* **Note 5**).
4. Carefully remove comb and assemble gel-running unit. Fill lower chamber with running buffer and remove any air-bubbles. Then fill upper chamber and rinse wells with running buffer with the help of a syringe.
5. Mix 4 µl sample with 1 µl 4x loading-buffer and heat 3 min at 95°C. Load gel immediately. Include one well with molecular weight markers.
6. Run the gel at 180 V until the dye-fronts reach the bottom of the gel.

3.3. Coomassie Staining

1. Remove the gel from the SDS-PAGE gel-running unit and separate the glass plates. Cut off and discard the stacking gel.
2. Remove the separating gel from the glass plate, place it into a small bowl and cover it with Coomassie staining solution. Put the gel onto a shaker and stain the gel for about 30 min.
3. Remove staining solution and add destaining solution to the gel. Destain the gel on the shaker for 2 h. Replace solution 2–3 times.

4. Put the gel on a Whatman-paper and dry it in a gel-dryer for 1 h (*see* **Fig. 2.1**).

3.4. Dot-Blot Analysis

1. Apply spots of 0.25, 0.5 and 1.0 µl of the biotinylated and purified target protein (*see* **Section 3.1**) onto a nitrocellulose membrane (4 × 5 cm in size). At the 1.0 µl spot first apply 0.5 µl and let dry for a minute. Then add the other 0.5 µl. This will prevent the spot from becoming too large. As a negative control apply spots of non-biotinylated protein. Cut off one edge of the membrane to ensure orientation.
2. Dry the membrane for 30 min in an incubator at 65°C.
3. Put the membrane into a small box and cover it with blocking buffer. Close the box and incubate the membrane for 3 h on a shaker (*see* **Note 6**).
4. Remove blocking buffer and wash the membrane in 1x PBS for 5 min.
5. Dilute the antibody 1:1,000 in blocking buffer and add this solution to the membrane. Wrap the box with the membrane in aluminium-foil. Incubate the membrane for 1 h on a shaker.
7. Remove the antibody solution and wash the membrane twice for 2 min in blocking buffer and then twice for 2 min in 1x PBS. Keep exposure to light as short as possible.
8. Remove the buffer and take the membrane out of the box with tweezers. Place the membrane onto a paper-towel. Place another towel on the membrane and dry the membrane

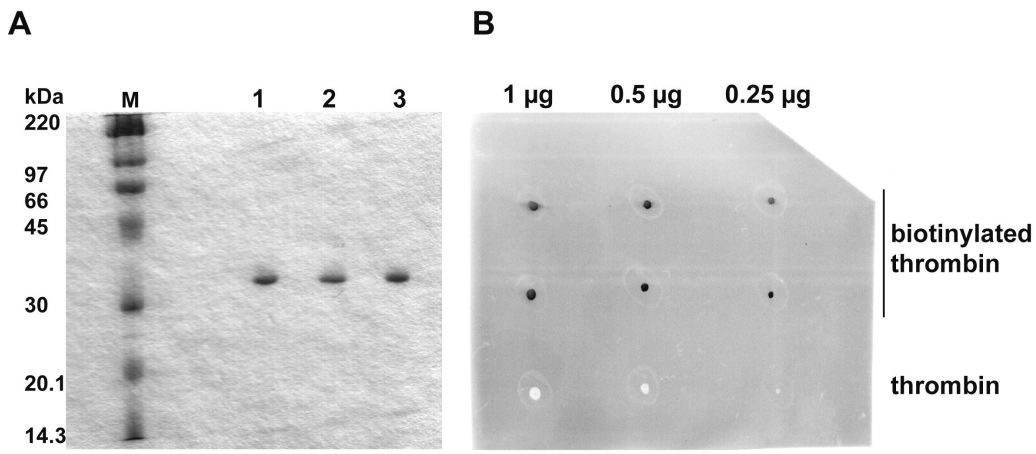

Fig. 2.1. Biotinylation of proteins. (**A**) 12% SDS-PAGE gel of human a-Thrombin after Coomassie staining. *M*: low range protein marker, *1*: 1 µg human α-Thrombin from stock, *2*: 1 µl biotinylation reaction before purification with P6 mirco-spin column, *3*: 1 µl biotinylated protein after purification with P6 micro-spin column. (**B**) Dot blot of human α-Thrombin. The indicated amounts of protein were spotted on a nitrocellulose membrane and were dried 30 min at 65°C and blocked 3 h in blocking buffer before incubation with monoclonal *anti*-biotin (mouse IgG1 isotype) FITC-conjugate.

between them by pressing the towels together with your hands. Do not wipe the membrane.

9. Place the membrane on a fluorescence-imager and read fluorescence (*see* **Fig. 2.1**).

3.5. Preparation of the Matrix

3.5.1. Pre-selection Matrix

1. Take 2.5 mg beads from stock. Separate the beads from storage buffer in a magnetic rack and discard supernatant.
2. Wash the beads twice with 250 µl 1x PBS and three times with 250 µl selection buffer. Resuspend the beads in 500 µl selection buffer.

3.5.2. Selection Matrix

1. Take 2.5 mg beads from the stock (10 mg/ml). Separate the beads from storage buffer in a magnetic rack and discard the supernatant.
2. Wash the beads twice with 250 µl 1x PBS and three times with 250 µl selection buffer. Resuspend the beads in 250 µl selection buffer.
3. Add 50 µl of the biotinylation reaction (*see* **Section 3.1**) to the prepared Dynabeads and incubate the suspension for 30 min at room temperature in a head-to-tail shaker.
4. Take off the supernatant and wash the beads twice with 250 µl selection buffer.
5. Resuspend the beads in 500 µl selection buffer. The beads can be stored at 4°C up to 1 week.

3.6. SELEX

3.6.1. First Cycle

1. Incubate 500 pmol of the ssDNA library in 80 µl selection with 80 µl of the pre-selection matrix (*see* **Section 3.5.1**) for 30 min at room temperature. Carefully resuspend the beads every 3 min by pipetting up and down.
2. Separate the beads in a magnetic rack and carefully transfer the supernatant to a new tube (*see* **Note 7**).
3. Incubate the supernatant with 80 µl selection matrix (*see* **Section 3.5.2**) for 30 min at room temperature. Carefully resuspend the beads every 3 min by pipetting up and down.
4. Separate beads in a magnetic stand and discard the supernatant. Resuspend the beads in two volumes (160 µl) of the selection buffer and incubate the resupension for 5 min. Separate beads and discard supernatant. Make sure that the buffer is completely removed.
5. Resuspend the beads in 100 µl water and heat for 3 min at 95°C.
6. Take off the supernatant and transfer it into a new tube. Discard the beads.

3.6.2. Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis

1. Prepare ten 100 µl PCR reactions: 1x PCR-buffer, 3.5 mM $MgCl_2$, 0.2 mM dNTP-mix, 1.1 µM forward primer, 0.9 µM reverse primer with 5' biotin-tag, 0.5 µg/µl BSA, 2.5 U Taq. Add water to reach 90 µl total volume (*see* **Note 8**).
2. Add 10 µl of the eluted ssDNA species (*see* **Section 3.6.1 Step 6**) to each PCR reaction and amplify the DNA in a thermocycler with the following settings: 1 min 95°C, 1 min 54°C, 1.5 min 72°C (*see* **Note 9**).
3. Prepare a 2.5% (w/v) agarose gel in 1x TBE-buffer and heat until the agarose is completely solved.
4. Add 0.1 µl/ml ethidiumbromide (10 mg/ml) and mix.
5. Pour the gel into a gel-casting chamber, insert the comb and let cool for 30 min.
6. Mix 2 µl PCR-product with 2 µl agarose-loading buffer and load the gel. Load one lane with a suitable DNA-ladder (e.g. 100 bp ladder).
7. Run the gel at 160 V with 1x TBE as running buffer for 20 min and visualize the dsDNA bands with a UV-lamp (λ = 254 nm).

3.6.3. Strand Displacement

1. Take 2.5 mg Dynabeads from the stock and remove storage buffer with the help of a magnetic rack. Wash the beads twice with 250 µl 1x B & W buffer.
2. Resuspend the beads in 500 µl 1x B & W buffer and add 500 µl 2x B & W buffer.
3. Add 500 µl PCR-product and incubate for 30 min at room temperature on a head-to-tail shaker (*see* **Note 10**).
4. Take off the supernatant and wash the beads three times with 250 µl 1x B & W buffer and once with 250 µl 2x B & W buffer.
5. Remove the buffer and resuspend the beads in 30 µl 0.15 M NaOH. Incubate for 3 min and separate beads using the magnetic rack.
6. Transfer the supernatant into a new tube and neutralize by adding 15 µl 0.3 M HCl. Control the pH by spotting a drop on pH-indicator paper with a pipette-tip and adjust pH with NaOH and HCl if necessary.
7. Add 16 µl 5x selection buffer and water to reach a total volume of 80 µl.

3.6.4. SELEX Cycles 2-x

1. Perform pre-selection and selection-step with the ssDNA following the instructions of the first SELEX cycle (*see* **Section 3.6.1**).
2. Raise the selection pressure by increasing the washing steps in each selection cycle: Wash two times in the second, four times in the third and eight times in the fourth cycle. Every four

washing steps the suspended beads can be incubated for 5 min at room temperature prior removal of the buffer. Reduce the amount of selection matrix used from the fifth cycle on (*see* **Note 11**).

3. After successful completion of the selection (monitoring of the selection can be accomplished by filter-retention analysis, *see* **Sections 3.7 and 3.9**) the library can be cloned and sequenced (Kits are available from various suppliers). A collection of representative sequences can be found in **Fig. 2.2**.

3.7. 5′-End Labelling of ssDNA Molecules

1. Take 200 µl of the PCR-product and perform the strand displacement using 100 µl streptavidin-coated beads. Elute ssDNA by adding 10 µl 0.15 M NaOH and neutralize the solution with 5 µl of 0.3 M HCl. Add water to reach a final volume of 20 µl.
2. Mix 10 µl ssDNA with 2 µl 10x PNK-Buffer. For control experiments prepare a second reaction using 10 pmol of the initial DNA library.
3. Add 2 µl of γ-[^{32}P]-ATP.
4. Add 20 U T4 polynucleotide-kinase. Add water to reach a total volume of 20 µl and incubate for 45 min at 37°C.
5. Add water to a final volume of 100 µl and purify the ssDNA by gel filtration using a G25 micro-spin column.

3.8. Polyacrylamide Gel Electrophoresis (PAGE)

1. Clean the glass plates with water and 70% ethanol before use.
2. Assemble the plates with the spacers and fasten them with several clips. Make sure the spacers are put neatly together without any gaps.
3. Prepare a 10% gel by mixing 20 ml concentrated gel solution, 25 ml thinner, 5 ml gel-buffer, 400 µl APS and 20 µl TEMED.
4. Pour the gel immediately and insert the comb. Lay the gel down horizontally and let polymerize for 45 min.
5. Remove the comb and wash away any gel fragments with water. Assemble the gel-running unit and fill the lower tank with 1x TBE. Remove any air-bubbles and fill the upper tank with 1x TBE.

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2.5      ACTACGGGGCTGGCGTGGTTGGGTAGTTGCTGGAGGAGAGCATCTTGAT

2.2      GGCTGGTGGGTTGGAGGGGTGCTCAAATATAATTTGGGTCGCTTTGGGT

1.3      GGTTGGTGTGGGTTGGATGGAGGCTGCGCCGTCACAGTATGCTGAGGAT

05.4     GATTACTCGCTCCCCGTGGTTGGCTTCGGGTTGGTCTGGGTGCGCTATT
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Fig. 2.2. Nucleic acid sequences of the DNA aptamers selected against human α-Thrombin 2.5, 2.2, 1.3 and 05.4, respectively. Shown are the initial random regions without the flanking primer regions.

6. Pre-run the gel 15 min at 380 V.
7. Mix 2 µl radioactive DNA with 18 µl loading buffer. Heat the samples to 95°C for 3 min.
8. Switch off the power and rinse the wells with 1x TBE with the help of a syringe.
9. Load the gel with each 15 µl of the samples.
10. Run the gel at 380 V with power limited to 25 W for about 1.5 h.
11. Take the gel out of the gel-running unit and remove one of the glass plates. Cover the gel with plastic foil.
12. Put the gel on a paper towel and put it in an X-ray film cassette.
13. Apply a phosphor-screen and close the cassette and expose the screen for 10 min. Analyse the screen using a phosphor-imager (e.g. Fuji FLA 3,000).

3.9. Filter-Retention Analysis

1. Dilute the radioactive labelled DNA 1:10 with water.
2. The diluted DNA is mixed 1:20 with binding-assay mix.
3. Prepare a concentration series of the target protein: Make a 2.5 µM solution in 1x PBS and dilute two times to 1.25 and 0.625 µM by mixing each one part protein solution with one part 1x PBS.
4. Mix in a well of a 96-well plate 20 µl of the prepared DNA in binding-assay mix with 5 µl protein solution. Prepare the samples for each protein-concentration at least in duplicate.
5. Cover the nitrocellulose membrane with 0.4 M KOH. Incubate samples and membrane for 20 min.
6. Assemble dot-blot unit. Equilibrate blotting-paper in binding-buffer and place it on the blotting unit. Take the membrane out of the KOH bath and shortly rinse it with water. Place it on top of the blotting-paper and remove any air-bubbles.
7. Complete the assembly of the unit and connect to vacuum pump.
8. Apply vacuum and wash the membrane twice with 200 µl binding-buffer using a multichannel-pipette. Drainage of the wells might be prevented by air-bubbles. To remove them, carefully tilt the blotting unit and tap it on the table.
9. With the help of a multichannel-pipette apply 20 µl of each sample on the membrane.
10. After filtration wash each well four times with 200 µl binding-buffer.
11. Disassemble the blot-unit and turn off the pump. Remove the membrane and dry it between paper towels.

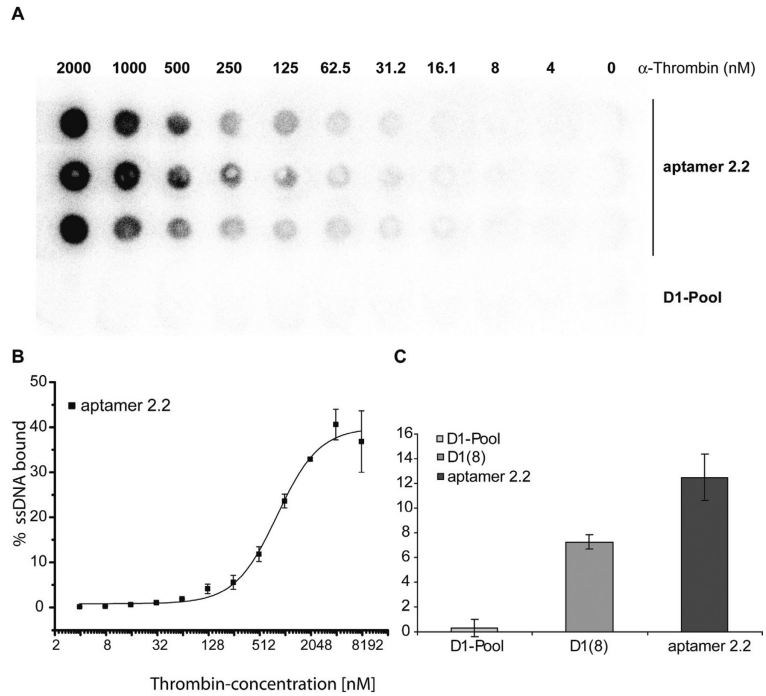

Fig. 2.3. Filter-retention analysis of the selected aptamer 2.2 after eight SELEX cycles. (**A**) Aptamer 2.2 was incubated with the indicated thrombin-concentrations for 20 min and filtrated through a nitrocellulose membrane (0.45 μm). The original D1-pool from which the aptamer has been selected shows no binding to thrombin. (**B**) Results from the filter-binding assays are evaluated with a non-linear logistic fit. The K_D-value of aptamer 2.2 was determined to be 816 nM. (**C**) Binding to human α-thrombin at 500 nM concentration. After eight SELEX cycles binding of the enriched pool (D1(8)) could be detected. Several sequences from the enriched pool were analysed of which aptamer 2.2 showed the highest affinity.

12. Place the membrane on a paper towel and cover it with foil. Cover the membrane with an X-ray film screen and close cassette.
13. Expose the screen for at least 1 h. Then take out the screen and read it in a phosphor-imager (*see* **Note 12,** *see* **Fig. 2.3**).

4. Notes

1. All solutions are prepared with de-ionized water purified with a resistivity of 18.2 MΩ and sterilized by filtration with a filter pore-size of 0.22 μm.
2. The D1-pool contains a random region consisting of 49 bases. We used this library in our lab with good results. DNA libraries with random regions are commercially available.
3. For the biotinylation the protein must not be solved in solutions containing Tris, as it reacts with the sulfo-NHS-LC-biotin.

Change buffer before the reaction. After the biotinylation Tris has no effect on the biotinylated protein.

4. Sometimes the biotinylation reaction can be inhibited by any impurities in the protein stock. If this is the case, purify the protein with a P6 spin-column before you add biotin to the reaction. P6 columns usually come in Tris storage-buffer, so be sure to change the buffer of the spin-column before use.
5. The blocking of the membrane in the dot blot can be reduced to 1 h to speed up the process, although this will lead to an increased background. For better quality block the membrane overnight.
6. SDS-PAGE-gels can be stored for 1–2 weeks at 4°C if you pack them in wet paper towels and wrap them in plastic foil.
7. The incubation with the pre-selection matrix removes sequences that bind to the matrix and not to the target.
8. A PCR-mastermix without DNA-polymerase can be prepared in advance and stored in aliquots at –20°C. The PCR-mix described here is optimized for the D1 library. Optimal concentrations of $MgCl_2$ and possible additives like BSA, glycerol or DMSO depend on the library and primers used.
9. Especially in the first SELEX cycle only a small fraction of the DNA library will bind to the target. Therefore, you may need much more PCR-cycles than usual. Control your product on an agarose gel and add more PCR-cycles if the product-yield is low, then check again. If you feel that your product is not amplified any more you may add fresh DNA-polymerase. The described PCR-conditions are optimized for the D1 library and depend on the used DNA library and primers.
10. The biotin-tag which has been inserted into the DNA by the PCR reaction binds to streptavidin. Thus, ssDNA can be eluted by denaturation while the counter-strand is restrained.
11. The number of SELEX cycles required for the selection of an aptamer varies from about 8 to 20 cycles and depends on the target and the DNA library.
12. Exposure-time of the binding-assay membrane to the X-ray film depends on the amount of radioactivity. In general, longer exposure leads to better contrast. Exposure overnight usually leads to good results.

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