Journal of Microbiology (2022) Vol. 60, No. 7, pp. 659–667
DOI 10.1007/s12275-022-2235-4
eISSN 1976-3794
pISSN 1225-8873

PROTOCOL

Development of DNA aptamers specific for small therapeutic peptides using a modified SELEX method

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(Received May 26, 2022 / Accepted May 27, 2022)

Aptamers are short single-stranded DNA or RNA oligonucleotides capable of binding with high affinity and specificity to target molecules. Because of their durability and ease of synthesis, aptamers are used in a wide range of biomedical fields, including the diagnosis of diseases and targeted delivery of therapeutic agents. The aptamers were selected using a process called systematic evolution of ligands by exponential enrichment (SELEX), which has been improved for various research purposes since its development in 1990. In this protocol, we describe a modified SELEX method that rapidly produces high aptamer screening yields using two types of magnetic beads. Using this method, we isolated an aptamer that specifically binds to an antimicrobial peptide. We suggest that by conjugating a small therapeutic-specific aptamer to a gold nanoparticle-based delivery system, which enhances the stability and intracellular delivery of peptides, aptamers selected by our method can be used for the development of therapeutic agents utilizing small therapeutic peptides.

Keywords: aptamer, antimicrobial peptide, multidrug-resistant bacteria, SELEX, magnetic beads, next-generation sequencing

Overview

Aptamers are short single-stranded DNA or RNA (ssDNA or ssRNA) molecules that can bind to specific targets, including proteins, peptides, carbohydrates, small molecules, toxins, and live cells, with high affinity and selectivity (for a recent review, see Zhuo *et al.*, 2017). The aptamer is usually compared with antibodies, owing to its target molecule binding characteristics, and has advantageous properties: it is more durable in living systems and easier to modify to improve stability and targeting ability, and has a longer half-life and lower or no immunogenicity. It has been used in various applications, including gene therapy, drug delivery, diagnostics, and biosensing (for a recent review, see Darmostuk *et al.*, 2015).

For identifying aptamers for molecules of interest, a method called systematic evolution of ligands by exponential enrichment (SELEX) has been used (Ellington and Szostak, 1990; Tuerk and Gold, 1990). The SELEX procedure generally consists of the following of steps (Aquino-Jarquin and Toscano-Garibay, 2011; Cibiel *et al.*, 2011; Kong and Byun, 2013): The nucleic acid library is mixed with the target material to form a complex, and the bound nucleic acids are collected from the unbound batch using separation material (partition). Thereafter, the complex-formed nucleic acids are eluted from the bound target to amplify the aptamer candidates (amplification), and the ssDNA is separated from the other strand to proceed to the next SELEX round (separation). These cycles are repeated until nucleic acid molecules with high binding affinities and specificities for the target are isolated.

In the SELEX procedure, extracting true aptamer molecules from non-specifically isolated nucleic acids is essential to successful selection. Since 1990, when SELEX technology was first introduced (Ellington and Szostak, 1990; Tuerk and Gold, 1990), various techniques have been developed to increase selection specificity. Generally, the traditional SELEX methods are based on "filtration" and "column purification," where the filtration method uses the nitrocellulose membrane as an affinity material and eliminates unbound nucleic acid molecules with a filter. The column purification method uses a column with an immobilized target that captures aptamer candidates in a passing nucleic acid library flow. are These techniques have limitations, such as the possibility of non-specific interaction of nucleic acids with affinity material that results in low yield and purity and the high demand for labor and time. In the attempts to overcome these limitations, modified versions of SELEX have been developed to increase the purity and yield of isolated aptamers and facilitate each SELEX step (Ellington and Szostak, 1992; Yang *et al.*, 2007).

When the target molecule is attached to an immobilization matrix, some sequences may undesirably interact with

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the matrix, adversely affecting the screening capability of SELEX. Negative SELEX (Ellington and Szostak, 1992) introduces the target molecule-unbound matrix in the middle of the SELEX round to remove the matrix-binding sequences. Another method, counter SELEX (Jenison *et al.*, 1994), increases aptamer selectivity by adopting a matrix coated with a material that has similar properties to those of the original target molecule. However, both methods require additional refinement steps, which lengthen the entire process.

Usually, the column-based SELEX method produces fewer specific aptamers because of the lack of a canonical binding position of a target molecule bound to the column matrix. For the arranging of the target molecules on the column matrix and exposing the same portion of the body, immunoaffinity SELEX uses an antibody-conjugated column matrix. However, maintaining the consistent affinity of antibodies from round to round is difficult (Setlem *et al.*, 2016).

A FluMag-SELEX using magnetic beads was devised to easily control the amount of target molecule in each round and conveniently observe the degree of aptamer separation. FluMag-SELEX was monitored while measuring the fluorescence activity of the aptamer amplified using 5′-modified primer polymerase chain reaction (PCR). However, the process of isolating ssDNA by gel elution after loading the amplified DNA onto the gel is time-consuming and results in a low yield (Stoltenburg *et al.*, 2005).

Because using sequence-target molecule complex binding materials is not completely free from non-specific bound nucleic acids, alternative techniques have been employed. For instance, capillary electrophoresis (CE-SELEX) separates aptamer-target substance complexes directly from the liquid phase. It allows aptamers to be selected in 2–4 round iterations by using the difference in movement speed between complex and non-binding sequences (Mendonsa and Bowser, 2004). CE-SELEX is faster than other SELEX methods that isolate aptamers through 5–15 repetitions; however, the system is suitable mostly for large molecules, such as proteins or lipopolysaccharides, because the mechanism of CE-SELEX relies on size separation by electrophoresis and the yield of selected aptamers is low, requiring further enrichment steps (Jing and Bowser, 2011).

As aforementioned, improved methods for different research purposes have been developed by inserting additional steps to decrease the proportion of non-specific sequences in the final aptamer pool or shortening the aptamer selection time. Two or more modified SELEX methods can be combined to identify aptamers with high affinities and selectivities. In this study, we developed a modified SELEX method using two types of magnetic beads (Fig. 1). Ni-NTA magnetic agarose beads were used to increase the selectivity of the aptamer for small target peptides. Streptavidin magnetic beads were used to facilitate the separation of aptamer candidates from the amplified dsDNA. The aptamers obtained through this process were selected by analyzing statistically significant abundant sequences via next-generation sequencing (NGS).

Application

Aptamer selection methods have been substantially improved as the new chemical synthesis of aptamers and the development of technical tools and analytical methods have become

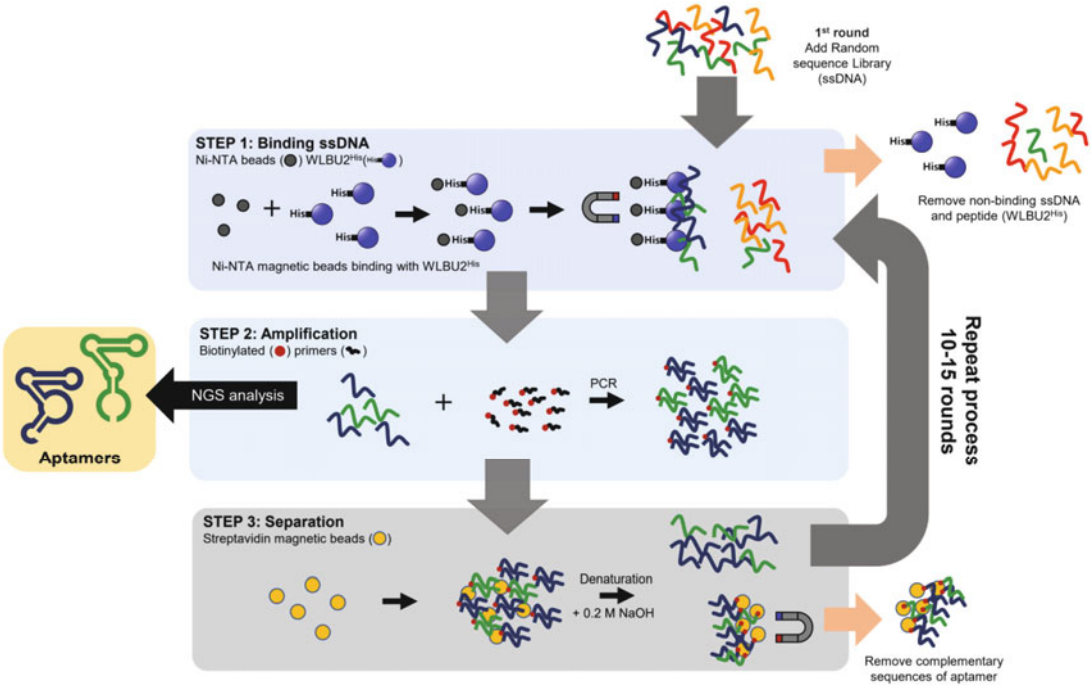

Fig. 1. Diagram of SELEX process. Step 1, Make a complex (ssDNA Library-WLBU2^{His}-Ni-NTA magnetic beads) by mixing the ssDNA library with WLBU2^{His}-Ni-NTA magnetic beads. Step 2, Amplify (PCR) the ssDNA sequences bound to AMP by using biotinylated primers. Step 3, Purify the amplified dsDNA with streptavidin magnetic beads, separate it into ssDNA with 0.2 M NaOH, and discard biotinylated strands with beads. After repeating a sufficient number of rounds, the final aptamer candidates were analyzed and selected using NGS analysis.

Table 1. Synthetic peptides and their minimum inhibitory concentrations (MICs)

Strain	Peptide	Sequence	M.W.	MIC[a] (μM)	References
S. Typhimurium	HPN3	RLEKLFSKIQNDK	1618.9	> 64	Yeom *et al.* (2016)
	HPN3^{His}	RLEKLFSKIQNDKHHHHHH	2549.8	> 54	
	A3-APO	RPDKPRPYLPRPRPPRPVR	2350.7	24–32	
	A3-APO^{His}	RPDKPRPYLPRPRPPRPVRHHHHHH	3173.6	32–40	
V. vulnificus	HPA3P	AKKVFKRLPKLFSKIWNWK	2417.2	8	Lee *et al.* (2017)
	HPA3P^{His}	AKKVFKRLPKLFSKIWNWKHHHHHH	3348.1	8	
	HPA3P2	AKKVFKRLPKLPSKIWNWK	2367	> 64	
	HPA3P2^{His}	AKKVFKRLPKLPSKIWNWKHHHHHH	3280.9	64	
	A3-APO	RPDKPRPYLPRPRPPRPVR	2350	> 64	
	A3-APO^{His}	RPDKPRPYLPRPRPPRPVRHHHHHH	3480.7	> 64	
A. baumannii	Lys AB2 P3	NPEKALEKLIAIQKAIKGMLNGWFTGVGFRRKR	3771.57	2–4	Park *et al.* (2022)
	Lys AB2 P3^{His}	NPEKALEKLIAIQKAIKGMLNGWFTGVGFRRKRHHHHHH	4594.42	2–4	
P. aeruginosa	WLBU2	RRWVRRVRRWVRRVVRVVRRWVRR	3400.1	4	In this study
	WLBU2^{His}	RRWVRRVRRWVRRVVRVVRRWVRRHHHHHH	4223	64–128	

[a] MIC of each peptide was determined using microdilution assays. The samples were incubated for 18–24 h at 37°C. After incubation, MICs were the lowest concentration of AMPs based on the OD_{600} value of the cultures.

available (for a recent review, see Blind and Blank, 2015). Aptamers can be developed as diagnostic tools, owing to their high affinity for their targets. For example, programmed death ligand 1 (PD-L1) is overexpressed in various cancer cells and is considered a potential biomarker for cancers overexpressing PD-L1. Because of the importance of PD-L1, an aptamer that targets this ligand was selected for cancer diagnosis (Yazdian-Robati *et al.*, 2017). In addition, aptamers have been widely applied in the diagnosis of ophthalmic and cardiovascular diseases, and cancer (Wan *et al.*, 2010; Song *et al.*, 2013; Darmostuk *et al.*, 2015). One of the most important applications of aptamers is their use in small-molecule therapeutics. Aptamers can act as antagonists by inhibiting protein-protein interactions by specifically binding to a target protein or as an agonist by increasing the delivery of therapeutic agents to target cells (Nimjee *et al.*, 2005; Zhou and Rossi, 2017). Aptamers can also be used as targeted drug delivery systems, primarily because of their specific binding to molecules or intended sites on target cells (Luo *et al.*, 2011). One example is the sgc8c DNA aptamer, which specifically targets T cell acute lymphoblastic leukemia cells. Doxorubicin covalently linked to an aptamer has been used therapeutically to specifically deliver the drug to target cells (Huang *et al.*, 2009).

Our research aim was to deliver antimicrobial peptides (AMPs) into animal cells using aptamers selected via a modified SELEX method. We used an aptamer as a linker for loading AMP on gold nanoparticles (AuNPs). AMP is a peptide with antimicrobial activity that has recently attracted attention for its effect on multidrug-resistant (MDR) bacteria (Ryu *et al.*, 2021). However, the use of AMPs as antimicrobial agents has been limited because they rapidly degrade when applied to animals and cannot usually penetrate animal cells infected with pathogens (Mahlapuu *et al.*, 2016). In overcoming these obstacles, DNA-functionalized gold nanoparticles (AuNPs) have been used as carriers (for a recent review, see Makabenta *et al.*, 2021). Studies have shown that this AuNP system can stably deliver C-terminally hexahistidine (6×His) tagged AMPs into organs of mice infected with *Salmonella enterica* serovar Typhimurium, *Vibrio vulnificus*, or *Acinetobacter baumannii*, resulting in the efficient clearance of these intracellular pathogens (Yeom *et al.*, 2016; Lee *et al.*, 2017; Park *et al.*, 2022). This method utilized a DNA aptamer specific for the 6×His tag that binds to 6×His-tagged AMPs. However, for certain AMPs, the addition of 6×His altered their structure and function (Table 1). Therefore, we attempted to develop a method to obtain DNA aptamers that specifically bind to small therapeutic peptides without 6×His or other tags.

Methods

SELEX

SELEX is divided into three major steps: complex formation (Ni-NTA magnetic agarose bead-DNA library-peptide), PCR

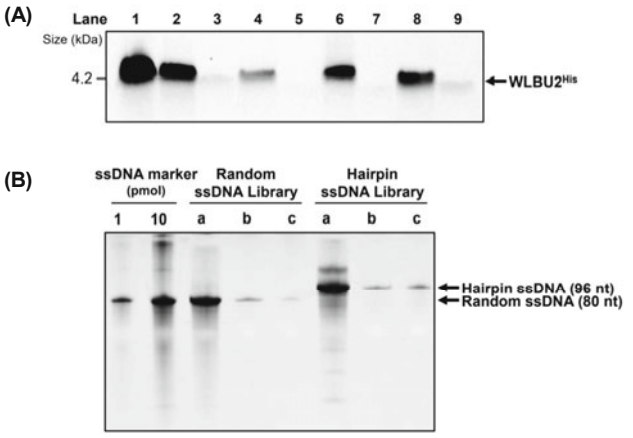

Fig. 2. Loading capacity of WLBU2^{His} and ssDNA on Ni-NTA magnetic agarose beads. (A) To assess loading capacity and elution efficiency of WLBU2^{His} with Ni-NTA magnetic agarose beads, we analyzed samples from each step by western blotting using his antibody. Lanes: 1, 0.2 nmol WLBU2^{His}; 2 and 6, unbound WLBU2^{His}; 3 and 7, WLBU2^{His} detached from beads after the wash step; 4 and 8, WLBU2^{His} eluted from beads; 5 and 9, WLBU2^{His} remaining on the bead after elution (Lanes 2 to 5 and 6 to 9 are from samples for random ssDNA library and hairpin ssDNA library, respectively). (B) Confirmation of binding ability between WLBU2^{His} bound to Ni-NTA magnetic agarose bead and ssDNA sequences with 12% denaturing acrylamide gel. Lanes: a, unbound ssDNA sequences; b, ssDNA sequences detached from WLBU2^{His}-Ni-NTA magnetic agarose beads after the wash step; c, Eluted ssDNA sequences.

(amplification), and ssDNA separation. In the formation of the complex, only aptamers that bind to the 6×His-tagged AMP ($WLBU2^{His}$), which has antibacterial activity against *Pseudomonas aeruginosa* (Deslouches *et al.*, 2007), were selected using Ni-NTA magnetic beads, and the aptamers that did not bind were discarded. Before the first round, the loading capacity of $WLBU2^{His}$ and ssDNA onto the Ni-NTA magnetic agarose beads was confirmed (Fig. 2). The first round used a random library of 2 nmol ssDNA, and the subsequent rounds used the total number of oligonucleotides selected in the previous round. Oligonucleotides were heated at 90°C for 10 min and cooled on ice for 5 min to unwind the secondary structure. The oligonucleotides and $WLBU2^{His}$ were combined in 1× PBS for 1 h. To pull down oligonucleotide-bound $WLBU2^{His}$, we mixed it with a Ni-NTA magnetic agarose bead mixture and incubated the resulting mixture for 1 h at RT. After pulling the complex magnetically and removing the supernatant, it was washed five times using wash buffer. The oligonucleotide-$WLBU2^{His}$ bound to beads were eluted using elution buffer, and oligonucleotides were purified through Phenol-chloroform DNA purification. As a negative control, all reactions were performed in the absence of $WLBU2^{His}$.

PCR was used to amplify eluted oligonucleotides. The PCR product was purified using the Favorprep Gel/PCR purification mini kit. The reverse primer contained biotin, and after PCR, the double strands were separated into single strands by using streptavidin beads.

Streptavidin beads were washed twice with 1× B&W buffer and incubated with the PCR products for 30 min. Non-biotinylated DNA strands were dissociated in 1× B&W buffer containing 0.2 M NaOH. The isolated ssDNA was desalted using ssDNA/RNA Clean & Concentrator. The specificity of the selected aptamer was improved by repeating these three steps several times. The last round was performed only up to the PCR. Selected and amplified DNA from each round of SELEX was confirmed by loading the gel in successive steps (Fig. 3).

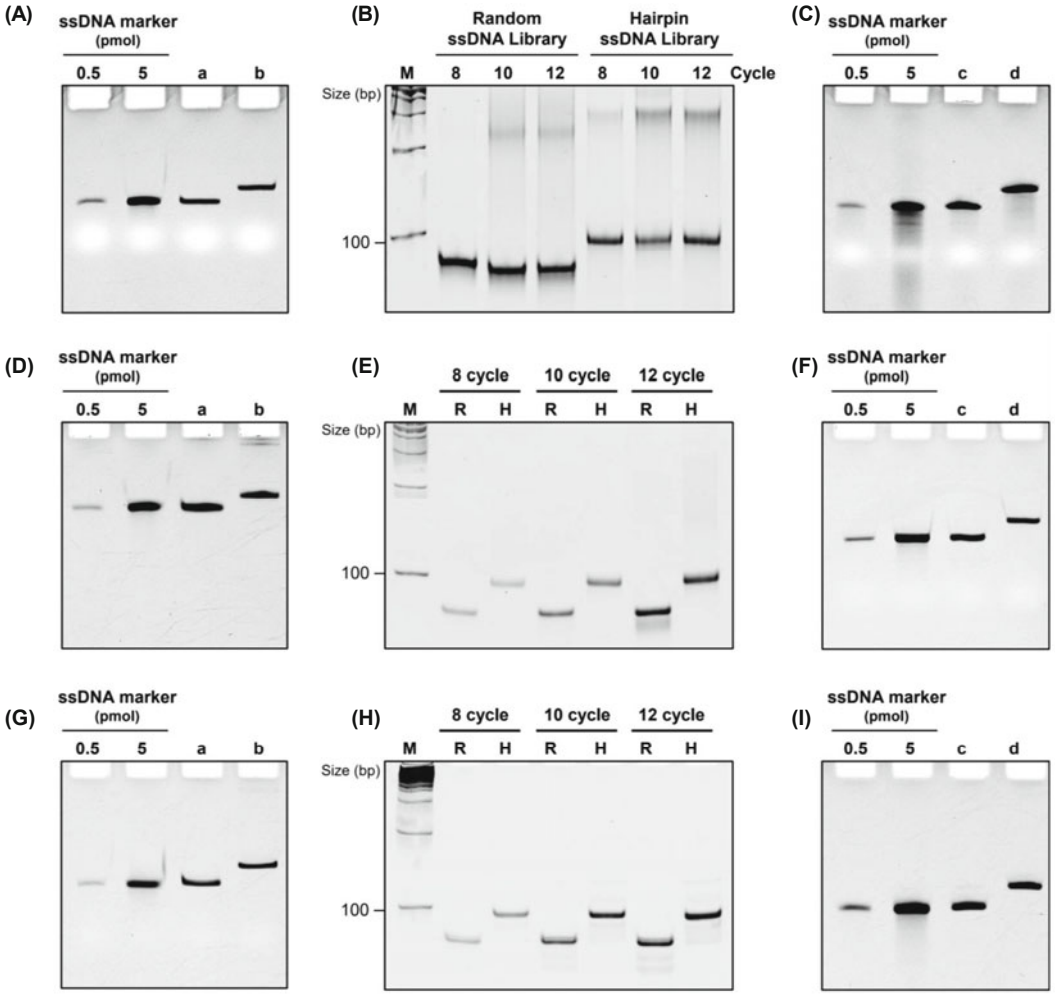

Fig. 3. Round process of binding, selection, and amplification of $WLBU2^{His}$-specific aptamers in single-stranded DNA libraries. (A–C): 1st round, (D–F): 5th round, (G–I): 11th round. (A, D, G) Confirmation of the amount of $WLBU2^{His}$-Ni-NTA magnetic agarose beads bound to ssDNA sequences with 12% denaturing acrylamide gel. Lanes: a, eluted random ssDNA sequences; b, eluted hairpin ssDNA sequences. (B, E, H) Eluted ssDNA sequences were amplified by PCR with non-biotinylated forward primers and biotinylated reverse primers. M, dsDNA size marker; R, random ssDNA library; H, hairpin ssDNA library. (C, F, I) PCR products were denatured with NaOH, and biotinylated ssDNA strands were pulled down with streptavidin magnetic beads. Aptamer candidates sorted from the random ssDNA library (lane c) and hairpin ssDNA library (lane d) were analyzed on a 12% denaturing acrylamide gel.

NGS

For the analysis of the sequences of the selected aptamers, NGS was performed using amplicon PCR, index PCR, and library normalization and pooling. Primers used for amplicon PCR contained the overhang adapter sequence required for compatibility with the Illumina index adapter. The PCR product was purified using the AMPure XP Bead, and DNA was dissolved in 10 mM Tris-HCl; pH 8.5. Index PCR was performed to add an index sequence so that various libraries could be pooled and sequenced. DNA was purified and dissolved using the same method used for amplicon PCR. Finally, the prepared samples were normalized and pooled. Normalization is the process of diluting each library present at different concentrations to the same concentration to ensure uniform reads for all samples. Each library was measured using qubits and diluted to 10 nM using 10 mM Tris-HCl; pH 8.5. A pooling library was created by mixing each library and denatured using 0.1 N NaOH. The pooled library and Phix control library were diluted with HT1 buffer to 1.3 pM and pooled at a Phix ratio of 30%. Sequencing was performed on the Illumina MiniSeq system using MiniSeq™ High Output Reagent Cartridge 150 cycles in paired-end mode.

NGS analysis

The Illumina paired-end reads were merged using PEAR v.0.9.11 (Zhang *et al.*, 2014) with the following parameters: quality 20 and timed length (bp) = 50. The assembled sequences were clustered using VSEARCH v.2.15.2 (Rognes *et al.*, 2016) with the following options: cluster fast mode, identity = 100%, identity definition = 1. The sample that would serve as a database in BLAST was indexed and compared with the database and queried with 95% identity using BLASTn v.2.10.0 (Camacho *et al.*, 2009).

Materials

Reagents

- Random library (5′-AAAACGACGGCCAGTN(50)CATGGTCATAGCTGT-3′,Bionics)
- Hairpin library (5′-AAAACGACGGCCAGTGCGCGCGCN(50)GCGCGCGCCATGGTCATAGCTGT-3′,Bionics)
- 10× PBS: 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4; pH 7.4
- Tween 20 (P1362.0500, Duchefa)
- Ni-NTA Magnetic Agarose Beads (36113, Qiagen)
- $WLBU2^{His}$ (RRWVRRVRRWVRRVVRVVRRWVRRHHHHHH, Anygen)
- Imidazole (56750, Sigma-Aldrich)
- Washing Buffer: 1× PBS, 10 mM imidazole, 0.04% Tween 20
- Elution Buffer: 1× PBS, 100 mM EDTA, 0.04% Tween 20
- GlycoBlue™ Coprecipitant (AM9516, Invitrogen)
- Sodium acetate (31215-0350, Junsei)
- Phenol:Chloroform:Isoamyl alcohol (25:24:1) (P2026, Biosesang)
- 100% EtOH (000E0219, Samchun)
- MG 2×-Hot master mix with dye (MP00505, MGmed)
- Favorprep Gel/PCR purification mini kit (FAGCK 001-1, Favorgen)
- Dynabeads™ MyOne™ Stereptavidin C1 (65001, Invitrogen)
- 2× B&W buffer: 10 mM Tris-HCl; pH 7.5, 1 mM EDTA, 2 M NaCl, 0.05% Tween 20
- ssDNA/RNA Clean & Concentrator™ (D7011, ZymoResearch)
- Qubit 1× dsDNA HS Assay (Q33230, Invitrogen)
- KAPA HiFi HotStart ReadyMix (KK2602, Roche)
- AMPure XP bead (A63881, BechmanCoulter)
- 1 M Tris-HCl; pH 8.5 (ML017-85, Welgene)
- 1 M Tris-HCl; pH 7.0 (ML017-70, Welgene)
- Sodium hydroxide solution (S2779, Sigma-Aldrich)
- MiniSeq™ High Output Reagent Cartridge 150 cycles (20000420, Illumina)
- Hyb Buffer (15027041, Illumina)

Primers

- M13 F (5′-AAAACGACGGCCAGT-3′, Bionics)
- M13 R (Biotin) (5′-ACAGCTATGACCATG-3′, Bionics)
- Amplicon F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAAACGACGGCCAGT-3′, Bionics)
- Amplicon R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACAGCTATGACCATG-3′, Bionics)
- Nextera XT Index Kit 24 indexes-96 samples (15055293, Illumina)

Equipment

- 1.5 ml Centrifuge tubes (SARSTEDT)
- 15 ml Conical tubes (SPL)
- PCR machine (Eppendorf)
- Centrifuge (Eppendorf)
- Vortexer (Bionex)
- Rotator (SLB)
- Nano drop (Nabi)
- Heat block (Wise Therm)
- DynaMag™-2 Magnet (12321D, Invitrogen)
- Qubit™ 4 Fluorometer, with WiFi (Q33238, Invitrogen)
- DynaMag™-96 Side Magnet (12331D, Invitrogen)
- MiniSeq® System (SY-410-1003, Illumina)

Hardware

- A computer with Unix, Linux or Mac OS X operating systems
- 4 CPU cores (recommend > 16 CPU cores & 2.1 GHz)
- RAM requirements: at least 4x file size (bytes). For instance, 1 Gigabytes (GB) of raw sequence file requires 4 GB of RAM (recommend > 32 GB)
- ~4x free disk space as raw sequence data (recommend > 100 GB) for storing output files

Software

- PEAR ver. 0.9.11 (https://cme.h-its.org/exelixis/web/ software/pear)
- VSEARCH ver. 2.21.1 (https://github.com/torognes/vsearch)
- BLAST ver. 2.13.0 (https://blast.ncbi.nlm.nih.gov)

Protocols

A. SELEX

- Step 1: Formation of complex (Ni-NTA Magnetic agarose bead-DNA library-Peptide)

1. Mix the 105 µl of T.D.W and 20 µl of 100 µM random library.
2. Heat at 90°C for 10 min and rapidly cool in ice for 5 min
3. Add 15 µl 10× PBS (final 1×) and 10 µl 50 µM $WLBU2^{His}$.
4. Incubate at room temperature for 1 h by using a rotator (Speed 7).
5. Prepare the bead mixture in a new centrifuge tube.
   - 10 µl Ni-NTA Magnetic agarose bead
   - 5 µl 10× PBS
   - 20 µl 100 mM imidazole
   - 2 µl 1% Tween 20
   - 13 µl T.D.W
6. Mix the mixture of 4 and 5.
7. Incubate at room temperature for 1 h by using a rotator (Speed 7).
8. Pull the Ni-NTA magnetic agarose beads by using a magnetic stand, and remove the supernatant.
9. Wash the bead by using 200 µl washing buffer and repeat 5 times.
10. Add 50 µl of elution buffer to beads and shake the tube by using a vortexer for 20 min.
11. Pull the Ni-NTA magnetic agarose beads by using a magnetic stand and the transfer the supernatant to a new centrifuge tube.
12. Repeat steps 10 and 11.
    Note: Merge the two eluted supernatants.
13. Purify aptamers using Phenol:Chloroform:Isoamyl alcohol (25:24:1).
    - 100 µl Elute sample
    - 300 µl T.D.W
    - 1 µl Glycoblute
    - 40 µl 3 M sodium acetate
    - 440 µl Phenol:Chloroform:Isoamyl alcohol (25:24:1)
14. Transfer the upper layer to a new centrifuge tube and centrifuge at 20,000 rpm for 10 min.
15. Next, add 440 µl phenol: chloroform: isoamyl alcohol (25:24:1) to the tube and vortex it for 1 min.
16. Transfer the upper layer to a new centrifuge tube, and centrifuge at 20,000 rpm for 10 min.
17. Add 1 ml 100% ethanol to the tube and vortex and incubate at -80°C for 1 h.
18. Centrifuge at 20,000 rcf for 20 min and remove the supernatant.
19. Add 500 µl 80% ethanol to the tube and vortex and incubate at -80°C for 1 h.
20. Centrifuge at 20,000 rcf for 20 min and remove the supernatant.
21. Dry the pellet under vacuum for 8 min, add 150 ul T.D.W to the pellet, and resuspend the pellet completely.

- Step 2: PCR

1. Prepare the PCR mixture.
   - 25 µl MG 2×-Hot master mix with dye
   - 10 µl Pre-stage DNA elute sample
   - 2 µl 10 µM M13 F primer
   - 2 µl 10 µM M13 R primer
   - 11 µl T.D.W
2. Set up the following PCR program.

STEP	TEMP	TIME
Initial denaturation	95°C	5 min
Denaturation	95°C	30 sec
Annealing	55°C	30 sec
Extension	72°C	30 sec
Final Extension	72°C	5 min

   Note: If the number of cycles is too high, other bands may be formed; thus, the appropriate number of cycles should be set so that only the target band can be formed.
3. Electrophoresis at 150 V for 40 min (10% polyacrylamide gel) to verify the PCR products.
4. After confirming the band of the appropriate intensity, PCR was performed with a total of 600 µl.
5. Clean up the PCR product by using the Favorprep Gel/PCR purification mini kit.
6. Elute the sample with 120 µl.

- Step 3: ssDNA separation

1. Wash the streptavidin beads with 100 µl of 1× B&W buffer, 3 times.
2. Add 100 µl of 2× B&W buffer and 100 µl of PCR product.
3. Incubate at RT for 30 min on a rotator.
4. Pull the streptavidin beads using the magnetic stand, Remove the supernatant.
5. Wash the Streptavidin beads with 200 µl of 1× B&W buffer 3 times.
6. Elute the beads with 50 µl of 1× B&W buffer (+ 0.2 M NaOH) at RT for 10 min on a vortexer 2 times.
7. Desalt the elute sample with ssDNA/RNA Clean & Concentrator™.
8. Elute the sample with 60 µl.

The selected ssDNA library was used for the next round of selection, and the same protocol was repeated 11 times. The final round proceeded to 2 step-PCR.

B. NGS

- Step 1: Amplicon PCR

1. Quantitate the DNA samples by using Qubit.
2. Equalize the concentration of DNA samples to 5 ng/µl.
3. Prepare the Amplicon PCR mixture.
   - 12.5 µl KAPA HiFi HotStart ReadyMix
   - 2.5 µl Pre-stagen DNA elute sample
   - 1 µl 10 µM Amplicon F primer
   - 1 µl 10 µM Amplicon R primer
   - 8 µl T.D.W
4. Set up the following PCR program:

STEP	TEMP	TIME
Initial denaturation	95°C	3 min
Denaturation	95°C	30 sec
Annealing	55°C	30 sec
Extension	72°C	30 sec
Final Extension	72°C	5 min

   Note: If the number of cycles is too high, other bands may be formed; thus, the appropriate number of cycles should be set so that only the target band can be formed.
5. Electrophoresis at 150 V for 20 min (2% agarose gel) to check the PCR product.

6. After confirming the band of appropriate intensity, mix the Amplicon PCR mixture and 40 µl AMPure XP beads.
7. Incubate at room temperature for 5 min.
8. Place the tube on a magnetic stand for 3 min and remove the supernatants.
9. Add 200 µl 80% EtOH, incubate the sample for 30 sec, and remove the supernatant.
10. Repeat step 9.
11. Dry at room temperature for 15 min.
12. Add 52.5 µl 10 mM Tris-HCl; pH 8.5 and mix gently.
13. Incubate at room temperature for 2 min.
14. Place the sample on a magnetic stand for 2 min and transfer 50 µl of the sample to a new tube.
15. Perform electrophoresis at 150 V for 20 min on a 2% agarose gel to verify the eluted DNA sample.

- Step 2: Index PCR

1. Prepare the Index PCR mixture.

25 µl	KAPA HiFi HotStart ReadyMix
5 µl	Pre-stage DNA elute sample
2.5 µl	Nextera XT Index Primer1 (N7xx)
2.5 µl	Nextera XT Index Primer2 (S5xx)
15 µl	T.D.W

2. Set up the following PCR program.

STEP	TEMP	TIME
Initial denaturation	95°C	3 min
Denaturation	95°C	30 sec
Annealing	55°C	30 sec
Extension	72°C	30 sec
Final Extension	72°C	5 min

Note: If the number of cycles is too high, other bands may be formed; thus, the appropriate number of cycles should be set so that only the target band can be formed.

3. Electrophoresis at 150 V for 20 min (2% agarose gel) to check the PCR product.
4. After confirming the band of appropriate intensity, mix the index PCR mixture and 50 µl AMPure XP beads.
5. Incubate at room temperature for 5 min.
6. Place the tube on a magnetic stand for 3 min and remove the supernatants.
7. Add 200 µl of 80% EtOH, incubate the sample for 30 sec, and remove the supernatant.
8. Repeat step 7.
9. Dry at room temperature for 15 min.
10. Add 27.5 µl 10 mM Tris-HCl; pH 8.5 and mix gently.
11. Incubate at room temperature for 2 min.
12. Place the tube on a magnetic stand for 2 min and transfer 25 µl of the sample to a new tube.
13. Electrophoresis was performed at 150 V for 20 min on a 2% agarose gel to verify the eluted DNA sample.

- Step 3: Library normalization and pooling

1. Quantitate the DNA samples by using Qubit.
2. Equalize the concentration of DNA samples to 10 nM using 10 mM Tris-HCl; pH 8.5.
3. Mix all DNA samples to create a pooled sequencing library.
4. Dilute the DNA sample to 1 nM using 10 mM Tris-HCl; pH 8.5.
5. Mix 5 µl 1 nM library and 5 µl 0.1 N NaOH.
6. Incubate at room temperature for 5 min.
7. Add 5 µl 200 mM Tris-HCl; pH 7.0 and 985 µl hybridization buffer.
8. Mix 5 µl 4 nM PhiX and 5 µl 0.1 N NaOH.
9. Incubate at room temperature for 5 min.
10. Add 5 µl 200 mM Tris-HCl; pH 7.0 and 985 µl hybridization buffer.
11. Dilute the library and PhiX to 1.3 pM using hybridization buffer.
12. Mix 350 µl 1.3 pM library and 150 µl 1.3 pM PhiX to prepare a pooled sequencing library.
13. Perform sequencing on the Illumina MiniSeq platform using MiniSeq™ High Output Reagent Cartridge 150 cycles in paired-end mode.

C. Analysis of Miniseq results

- Step 1: Data preparation

1. Set up a working directory, which is the folder location where the input and output files are stored and generated.
2. Unzip the selex files in each sample folder.
   % gzip -d file
3. Use the PEAR (ver. 0.9.11) tool to pair forward and reverse sequence reads and merge them into one sequence.
   % cd Sample_#
   % mkdir pear
   % pear -f forward sample -r reverse sample -o ./pear/#_RF.fq -t 50 -q 2o -j 30
4. Convert files from fq format to fa format
   % cd pear
   % fq2fa #_FR.fq.assembled.fastq #_RF.fa

- Step 2: Clustering and Blast

1. Using VSEARCH v.2.21.1, we selected the identity ratio to cluster the sequence.
   % cd Sample_#
   % mkdir vsearch
   % cp pear/#_RF.fa Sample_#/vserach
   % cd vsearch
   % vsearch --cluster_fast #_RF.fa --id 1.00 --strand plus --sizeout --fasta_width 0 —centroids #_RF_derep_centroids_100_id1.fa --msaout #_RF_MSA_100_id1.txt —threads 14 --iddef 1
2. Check the size of the oligonucleotides of the same sequence and delete those with a size of 10 or less.
   % vsearch –sortbysize #_RF_derep_centroids_100_id1.fa –output #_RF_size_100_id1.fa
3. Conduct BLAST by using BLASTn v.2.10.0, with a database and query identity ratio of 95.
   % cd Sample_#
   % mkdir Blast
   *% blastn -query __ -db __ -out __ *
   *-outfmt "6 qseqid sacc pident qcovs qlen length evalue bitscore" *
   *-max_target_seqs 1000 -num_threads 8 -perc_identity 95 *
   -qcov_hsp_perc 95 -word_size 4

Expected Results

Our suggested SELEX protocol not only facilitates the control of the substances used in the experiment but also efficiently shortens the aptamer screening time than the FluMag-

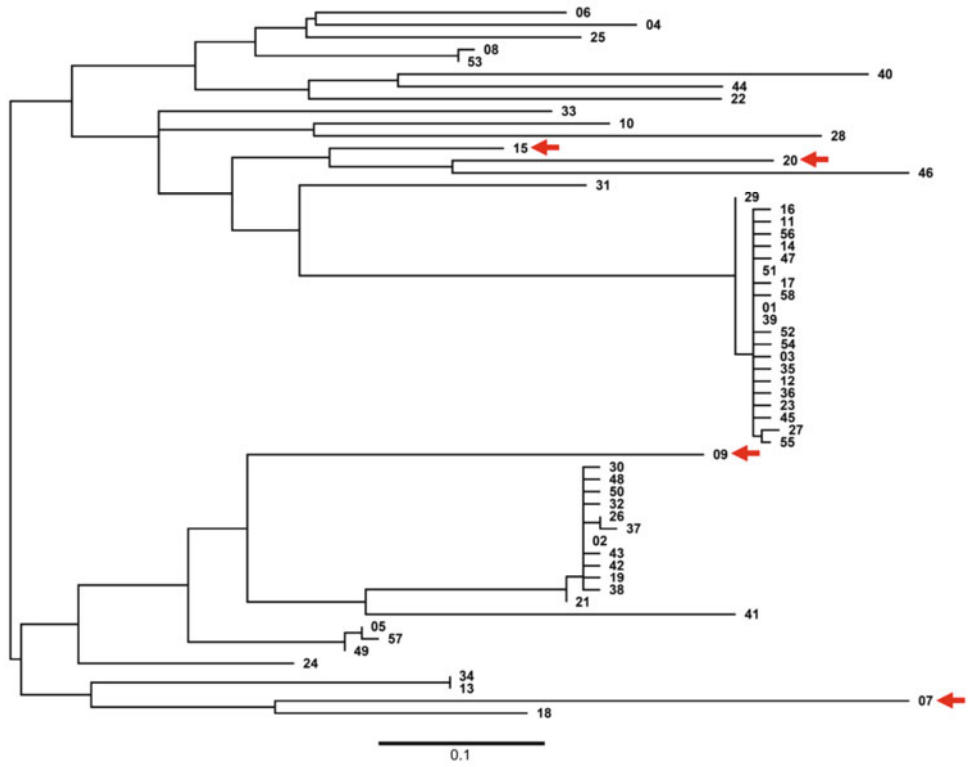

Fig. 4. Phylogenetic tree using NGS reads from aptamer libraries. The tree was reconstructed using the maximum likelihood method. Aptamer reads with > 0.05% of the total reads were used. The four representative consensus sequences were screened using the SELEX system and are shown separately.

SELEX. In addition, statistically meaningful aptamer sequences can be selected through NGS analysis, and individual aptamer synthesis is possible because the information on each sequence is known. Using this modified SELEX method, aptamers can be selected more quickly and accurately compared to other conventional SELEX methods, which can accelerate the screening of therapeutics and biosensors.

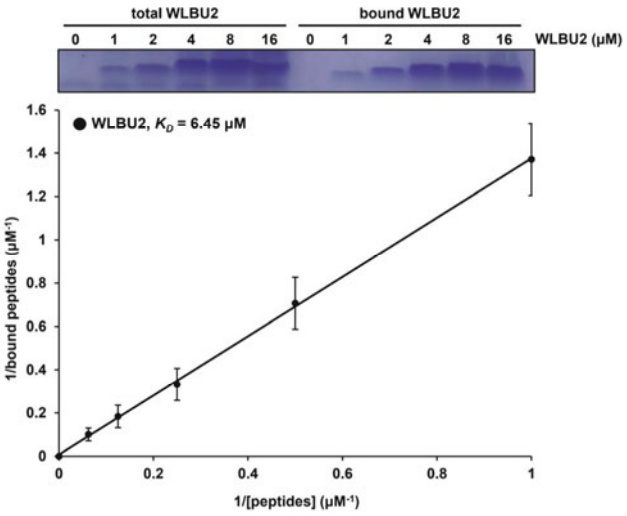

Fig. 5. Binding capacity between AuNP-Apt^{WLBU2} and WLBU2. The binding capacity of AuNP-Apt^{WLBU2} for WLBU2 was determined. The indicated concentrations (0, 1, 2, 4, 8, and 16 μM) of WLBU2 were incubated with AuNP-Apt^{WLBU2} (5 nM) by mixing, followed by incubation for 10 min. AuNP-Apt^{WLBU2}-bound WLBU2 (bound WLBU2) was analyzed by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) and Coomassie Brilliant Blue staining. The amount of WLBU2 bound to AuNP-Apt^{WLBU2} was analyzed by quantifying band intensities and compared with the standards where known amounts of WLBU2 (total WLBU2) were electrophoresed. The dissociation constants (K_D) were obtained from the slopes of the graphs.

In this study, aptamers that specifically bound to WLBU2 were selected according to the SELEX protocol. The aptamer pool selected for WLBU2 from a random DNA library was analyzed using NGS to obtain a sequence with 436,825 reads. Only high-repetition sequences (> 10-times repeat reads) were analyzed, excluding low-repetition sequences, resulting in sequences with 877 reads. The phylogenetic tree was used 86.33% in the total reads. Many repetitive sequences were clustered and showed the orders of the highest ratio (01 to 58) (Fig. 4). WLBU2-specific binding aptamer occupies more than 0.18% of the analyzed sample and it was defined as having a value more than twice the ratio in the non-specific random DNA library. Four representative nucleotide sequences satisfying this condition were shown as candidate nucleotide sequences selected by SELEX (07, 09, 15, and 20 in Fig. 4). One of the candidate sequences was synthesized as an aptamer and was used to assess the binding constant of the aptamer to WLBU2. Under the conditions used in this binding capacity assay, the binding constant was estimated to be 6.45 μM (Fig. 5), indicating that DNA aptamers that can specifically bind to small peptides can be selected using our modified SELEX method.

Our findings show that the modified SELEX method can be used to select DNA aptamers specific to small therapeutic peptides.

Acknowledgements

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (IPET) through Animal Disease Management Technology Advancement Support Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) [grant number: 122060-02-1-CG000 to J. B.] and the National Research Foundation of Korea [NRF-2021R1A2C3008934 to K. L]. This research was also supported by Chung-Ang University Graduate Research Scholarship grants in 2020.

Conflict of Interest

The authors declare no conflict of interest.

References

Aquino-Jarquin, G. and Toscano-Garibay, J.D. 2011. RNA aptamer evolution: two decades of SELEction. *Int. J. Mol. Sci.* **12**, 9155–9171.

Blind, M. and Blank, M. 2015. Aptamer selection technology and recent advances. *Mol. Ther. Nucleic Acids* **4**, e223.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T.L. 2009. BLAST+: architecture and applications. *BMC Bioinformatics* **10**, 421.

Cibiel, A., Dupont, D.M., and Ducongé, F. 2011. Methods to identify aptamers against cell surface biomarkers. *Pharmaceuticals* **4**, 1216–1235.

Darmostuk, M., Rimpelova, S., Gbelcova, H., and Ruml, T. 2015. Current approaches in SELEX: an update to aptamer selection technology. *Biotechnol. Adv.* **33**, 1141–1161.

Deslouches, B., Gonzalez, I.A., DeAlmeida, D., Islam, K., Steele, C., Montelaro, R.C., and Mietzner, T.A. 2007. *De novo*-derived cationic antimicrobial peptide activity in a murine model of *Pseudomonas aeruginosa* bacteraemia. *J. Antimicrob. Chemother.* **60**, 669–672.

Ellington, A.D. and Szostak, J.W. 1990. *In vitro* selection of RNA molecules that bind specific ligands. *Nature* **346**, 818–822.

Ellington, A.D. and Szostak, J.W. 1992. Selection *in vitro* of single-stranded DNA molecules that fold into specific ligand-binding structures. *Nature* **355**, 850–852.

Huang, Y.F., Shangguan, D., Liu, H., Phillips, J.A., Zhang, X., Chen, Y., and Tan, W. 2009. Molecular assembly of an aptamer-drug conjugate for targeted drug delivery to tumor cells. *Chembiochem* **10**, 862–868.

Jenison, R.D., Gill, S.C., Pardi, A., and Polisky, B. 1994. High-resolution molecular discrimination by RNA. *Science* **263**, 1425–1429.

Jing, M. and Bowser, M.T. 2011. Isolation of DNA aptamers using micro free flow electrophoresis. *Lab Chip* **11**, 3703–3709.

Kong, H.Y. and Byun, J. 2013. Nucleic acid aptamers: new methods for selection, stabilization, and application in biomedical science. *Biomol. Ther.* **21**, 423–434.

Lee, B., Park, J., Ryu, M., Kim, S., Joo, M., Yeom, J.H., Kim, S., Park, Y., Lee, K., and Bae, J. 2017. Antimicrobial peptide-loaded gold nanoparticle-DNA aptamer conjugates as highly effective antibacterial therapeutics against *Vibrio vulnificus*. *Sci. Rep.* **7**, 13572.

Luo, Y.L., Shiao, Y.S., and Huang, Y.F. 2011. Release of photoactivatable drugs from plasmonic nanoparticles for targeted cancer therapy. *ACS Nano* **5**, 7796–7804.

Mahlapuu, M., Håkansson, J., Ringstad, L., and Björn, C. 2016. Antimicrobial peptides: an emerging category of therapeutic agents. *Front. Cell. Infect. Microbiol.* **6**, 194.

Makabenta, J.M.V., Nabawy, A., Li, C.H., Schmidt-Malan, S., Patel, R., and Rotello, V.M. 2021. Nanomaterial-based therapeutics for antibiotic-resistant bacterial infections. *Nat. Rev. Microbiol.* **19**, 23–36.

Mendonsa, S.D. and Bowser, M.T. 2004. *In vitro* selection of high-affinity DNA ligands for human IgE using capillary electrophoresis. *Anal. Chem.* **76**, 5387–5392.

Nimjee, S.M., Rusconi, C.P., and Sullenger, B.A. 2005. Aptamers: an emerging class of therapeutics. *Annu. Rev. Med.* **56**, 555–583.

Park, J., Shin, E., Yeom, J.H., Choi, Y., Joo, M., Lee, M., Kim, J.H., Bae, J., and Lee, K. 2022. Gold nanoparticle-DNA aptamer-assisted delivery of antimicrobial peptide effectively inhibits *Acinetobacter baumannii* infection in mice. *J. Microbiol.* **60**, 128–136.

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. 2016. VSEARCH: a versatile open source tool for metagenomics. *PeerJ* **4**, e2584.

Ryu, M., Park, J., Yeom, J.H., Joo, M., and Lee, K. 2021. Rediscovery of antimicrobial peptides as therapeutic agents. *J. Microbiol.* **59**, 113–123.

Setlem, K., Mondal, B., Ramlal, S., and Kingston, J. 2016. Immuno affinity SELEX for simple, rapid, and cost-effective aptamer enrichment and identification against aflatoxin B1. *Front. Microbiol.* **7**, 1909.

Song, Y., Zhu, Z., An, Y., Zhang, W., Zhang, H., Liu, D., Yu, C., Duan, W., and Yang, C.J. 2013. Selection of DNA aptamers against epithelial cell adhesion molecule for cancer cell imaging and circulating tumor cell capture. *Anal. Chem.* **85**, 4141–4149.

Stoltenburg, R., Reinemann, C., and Strehlitz, B. 2005. FluMag-SELEX as an advantageous method for DNA aptamer selection. *Anal. Bioanal. Chem.* **383**, 83–91.

Tuerk, C. and Gold, L. 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**, 505–510.

Wan, Y., Kim, Y., Li, N., Cho, S.K., Bachoo, R., Ellington, A.D., and Iqbal, S.M. 2010. Surface-immobilized aptamers for cancer cell isolation and microscopic cytology. *Cancer Res.* **70**, 9371–9380.

Yang, Y., Yang, D., Schluesener, H.J., and Zhang, Z. 2007. Advances in SELEX and application of aptamers in the central nervous system. *Biomol. Eng.* **24**, 583–592.

Yazdian-Robati, R., Ramezani, M., Khedri, M., Ansari, N., Abnous, K., and Taghdisi, S.M. 2017. An aptamer for recognizing the transmembrane protein PDL-1 (programmed death-ligand 1), and its application to fluorometric single cell detection of human ovarian carcinoma cells. *Microchim. Acta* **184**, 4029–4035.

Yeom, J.H., Lee, B., Kim, D., Lee, J., Kim, S., Bae, J., Park, Y., and Lee, K. 2016. Gold nanoparticle-DNA aptamer conjugate-assisted delivery of antimicrobial peptide effectively eliminates intracellular *Salmonella enterica* serovar Typhimurium. *Biomaterials* **104**, 43–51.

Zhang, J., Kobert, K., Flouri, T., and Stamatakis, A. 2014. PEAR: a fast and accurate Illumina paired-end reAd mergeR. *Bioinformatics* **30**, 614–620.

Zhou, J. and Rossi, J. 2017. Aptamers as targeted therapeutics: current potential and challenges. *Nat. Rev. Drug Discov.* **16**, 181–202.

Zhuo, Z., Yu, Y., Wang, M., Li, J., Zhang, Z., Liu, J., Wu, X., Lu, A., Zhang, G., and Zhang, B. 2017. Recent advances in SELEX technology and aptamer applications in biomedicine. *Int. J. Mol. Sci.* **18**, 2142.