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Identification of Aptamers that Specifically Bind to A₁ Antigen by Performing Cell-on Human Erythrocytes

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Abstract

Background: The apply of aptamers as a new generation's way to probe diagnostic for the detection of target molecules has gained ground. Aptamers can be used as alternatives to diagnostic antibodies for detection of blood groups due to their unique features. This study was aimed to produce DNA diagnostic aptamer detecting the antigen of A₁ blood group using the Cell-Selex method. **Materials and Methods:** DNA aptamer was isolated against A₁ RBC antigen after ten stages of Cell-Selex and amplification by an asymmetric polymerase chain reaction. The progress of the stages of selection was evaluated using flow cytometry analysis, which the DNA aptamer isolated from the tenth cycle with an affinity of 70% fluorescent intensity, was selected from four positive colonies followed by determination of the sequences and secondary structures. **Results:** The aptameric sequence obtained from C₄ cloning was calculated with the highest binding affinity to A₁ antigen having an apparent dissociation constant (K_d value) of at least 29.5 ± 4.3 Pmol, which was introduced as the selected aptamer-based on ΔG obtained from a colony of C₄ equal to -13.13 . **Conclusion:** The aptamer obtained from using Cell-Selex method could be used as an example for the development of diagnostic tools such as biosensors for detecting A₁ blood group antigens. [GMJ.2020;9:e1657] DOI:[10.31661/gmj.v9i0.1657](https://doi.org/10.31661/gmj.v9i0.1657)

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Introduction

One of the first and most basic tests in transfusion bank is to determine the grouping of antigens on red blood cell (RBC) surface based on the ABO blood group system. The results of the ABO blood group system are effective in determining the transfused blood

type and conducting transplantation of solid organs. Nowadays, the ABO blood group antigens are recognized by serological methods using antibodies [1, 2]. Serological methods for determining blood groups have some problems, including inconsistency of results of cell and serum grouping, technical errors, presence of alloantibodies, and other disad-

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vantages and limitations of using antibodies (e.g., limitations of polyclonal and monoclonal antibodies, high costs, prolonged and delicate production process, difficulty in storage and transportation, and the need for specific situations) [3, 4]. All this leads to the development of strategies aimed at producing an alternative structure that can be easily chosen, manufactured, and manipulated by standard biomolecular techniques [5, 6]. One of the new tools are aptamers that can have a better activity than antibodies in the diagnosis, control, and drug delivery as novel biomarkers both in vitro and in vivo [6]. Aptamers are short-chain, single-stranded DNA or RNA with a length of 90–100 nucleotides. They can bind to targets including protein, peptides, amino acids, ATP, antibodies, small chemical molecules, viruses, whole cells, cellular receptors, antigens, metal ions, and enzymes based on their complex 3-D constructs. They have high specificity and binding affinity as well as dissociation constants in the range of nano- and picomolar [5]. Production, development, and replacement of aptamers with monoclonal antibodies are important for comparative functional advantages. The basic parameters of aptamers include the availability of raw materials, less complicated production methods, easy amplification and storage along with long shelf life, less technical problems and complexity, low cost, high purity, good quality, stability, and high diversity [7]. Short oligonucleotide fragments of single-stranded DNA or RNA are synthetically made in vitro using combinatorial chemistry techniques. The aptamers then target nearby molecules with pre-designed specific primers by a mode of selection called systematic evolution of ligands by exponential enrichment (SELEX), and then they are amplified by polymerase chain reaction (PCR) through washing process [8]. Following amplification, the dsDNA aptamer converts into a single-stranded one by asymmetric PCR techniques or via lambda exonuclease, which is prepared for the rest of the process [9]. Selex cycles are sequentially repeated between 6 to 20 times to yield a nucleic acid ligand with the highest affinity to the target molecule, after which the process ceases, the selection and progress processes are monitored by screening tools like flow

cytometry and ELISA to achieve the highest affinity [10, 11]. Later, Birch *et al.* cloned the ligand to determine the sequence in a specified vector with the aim of drug delivery [9, 12]. He identified aptamers of RBC surface proteins infected with aptameric malaria mosquitoes. Duan *et al.* developed a whole-bacterium SELEX technique to select aptamers that specifically bound to *L. monocytogenes* with high affinity [13]. Dwivedi *et al.* provide proof-of-concept that biotinylated aptamers selected by whole-cell SELEX can be used in a qPCR-based capture-detection platform for *Salmonella Typhimurium* [14]. Kazunori Ikebukuro *et al.* carried out pre-selection of PSA-binding aptamers by SELEX to obtain candidates for post-SELEX screening using GA and obtained five aptamers that bound to PSA [15]. However, no study was found to determine blood groups using aptamers. Given that most blood group variations are observed in blood group A and its subset A₁ [16]. So, this study aimed to identify and locate DNA aptamers with a high specific affinity to A₁ RBC using Cell-Selex approach and asymmetric amplification, which selected aptamers were confirmed by flow cytometry analysis followed by sequencing and prediction of their secondary structures that can replace antibodies.

Materials and Methods

An amount of 1×10^6 antigens are present on the surface of human RBCs. A₁ RBC was obtained from the blood transfusion in sterilized bags with a refrigerated shelf life of 35 days. For each use within 24 h, 9.6 ml of blood with 0.4 ml of 20% citrate solution was poured in the falcon and then centrifuged at 1000 g for 20 minutes. After pelleting RBCs and removal of plasma and WBCs, RBCs were washed with PBS phosphate buffer salt three times (10 minutes per wash) at 1000 g. In order to prepare a stock or flow cytometry analysis, the cells were first examined for survival and activity, and then counted using a hemocytometer. Primers were designed depending on the conditions and requirements with help of the online tool Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and Gene runner Software version 6.5.50 (<http://>

www.generunner.net). The forward primer was designed in a size of 21 nucleotides with the sequence 5'-CATCCATGGGAATTC-GTCGAC-3' and a temperature of 59.13°C. The reverse primer was constructed by 19 nucleotides with the sequence 5'-CTTCCTA-AGCTCGATCTCG-3' at 54.65 °C. The primers were ordered and prepared in two forms of the simple and fluorescent tag (fluorescein bound to 5' end) for flow cytometry analysis. The length of 38 nucleotides was meant for the randomized region. Accordingly, a synthetic single-stranded DNA library was designed with a length of 78 nucleotides in the two fixed (determinate) and variable (indeterminate and random) sections having HPLC-grade purity, and it was purchased from Metabion Company, Germany. The PCR reaction substances including Taq DNA polymerase, PCR 10x buffer, MgCl₂ solution, 1500 mM KCl, and 200 mM Tris-HCl were purchased from Sina Clone Company, Iran. TEA10X buffer (1X buffer contains 40 mM Tris, 2 mM acetic acid, and 1 mM EDTA) was purchased from Fermentas Company, Canada. DNA marker and loading paint 6X bromophenol blue-xylene cyanol were obtained from Geneone (Germany) and Uantis (USA), respectively. The safe stain paint (a kind of cyber green paint) and molecular grade agarose were obtained from Sina Clones, Iran. The water for PCR reaction was purified with a Millipore system (USA). The A₁ polyclonal antibody for Counter Selex was purchased from Thermo Fisher Scientific, USA. TA Cloning vector and plasmid purification kits were purchased from INTRON (Seoul, South Korea). The gel extraction kit was obtained from Bioneer, South Korea.

Production of a Single-stranded Library

A synthetic library with 78-mer nucleotides was amplified by ten cycles of PCR. The 4-step thermal program included an initial denaturation (5 min, 95 °C, one cycle), the main denaturation (35 secs, 95°C), annealing (35 secs, 62 °C), and elongation (3 min, 72 °C), which were repeated in 35 cycles. A final elongation step was performed in one cycle at 72 °C for 3 minutes. The amplification process was carried out using primers F and R. The ssDNA was converted into dsDNA during the addition of asymmetric PCR (35 cycles) using

primer F only as the dominant primer with a ratio of 20:1 at a final volume of 30µl. PCR products were loaded on a 2% agarose gel and stained with cyber green color (1:10.000). Electrophoresis was performed using a Multi-sub horizontal tank (Clear Company, UK) in 80 V at room temperature for 30 minutes. The products of PCR amplification (10 µl) were loaded on an agarose gel with 2 µl of the buffer paint and evaluated by GelDoc device. The Gene Tools software (Syngene, Syngene UK, Cambridge, UK, <http://www.syngene.com/syngene-uk/>) was used for image capture and analysis of DNA spots.

Aptamer Selection Process (Cell-Selex)

After determining cell viability, 500 µl of binding buffer (PBS containing magnesium and calcium) with 1 µl of the single-stranded library was added to the ready cell pellet and placed at 4 °C with gentle shaking (150 rpm) for 10 minutes. With the progress of enrichment stages, the incubation time was reduced from 1h to 30 minutes. To remove the raw unannealed sequence in each round of selection, the pellet was centrifuged (8000 rpm, 10 minutes) and then rinsed with washing buffer. Then, the cell pellet obtained from Selex was dissolved in DNase distilled water (100 ml) and incubated (70 °C, 10 min) for denaturation. The cells were then deposited by centrifugation (13000 rpm, 30 sec), and the supernatant containing aptamers attached to the cells were collected, and amplified by PCR. To limit the aptamers capable of attaching, Counter Selex was carried out on the masked RBC cells with A₁ polyclonal antibody at the end of the third, fifth, and seventh cycles [16].

Flow Cytometry Analysis

After three selection processes of DNA aptamer attached to the RBCs, the fluorescent intensity was measured at the 10th, 9th, 8th, 6th, and 4th cycles of the selection process (Rooyan Institute, Iran) to track enrichment of the selected library. First, the single-stranded aptamer labeled with FITC (50 P moles) was added to the binding buffer (100 ml), heated at 95 °C for 5 minutes, and placed on ice for 5 minutes. The aptamers were added to the RBC pellet from the Selex and incubated at 4 °C for 45 minutes. After incubation, the cells

were washed twice and analyzed by flow cytometry.

Cloning, Sequencing, and Structural Analysis of Selected Aptamers

After 10 stages of the selection process and achieving the best results for fluorescent intensity in the 10th Selex cycle, the enriched aptamer DNA with a high binding affinity was selected, amplified with primers, cloned into PBluscript vector, and transferred to the genetically modified *E. coli* tpo10 cells. White colonies were collected on LB medium (Luria-Bertani, with ampicillin at 37 °C) after 24 h. Then, colonies were confirmed by Colony-PCR method using aptamer-specific simple primers followed by selection of positive colonies for evaluation and characterization. Positive colonies containing aptamer sequences were amplified with primers labeled with FITC, and the fluorescence intensity was measured using flow cytometry analysis. The aptamers showing the highest binding affinity to A₁ RBC antigen was selected for sequencing.

Determining the Secondary Structures of Selected Sequence

The secondary structures of the selected aptamers were analyzed and predicted by the Mfold online software version 3.5 (<http://Unafold.rna.albany.edu/Q=Mfold>) under conditions of Na⁺ [144 mmol/L] and Mg²⁺ (0.4

mmol/L) at 21 °C [17].

Determining the Dissociation Constant

For evaluation of the dissociation constant or binding of fluorescent aptamers to A₁ RBC, different concentrations of aptamers labeled with FITC were incubated at room temperature with gentle shaking for 45 minutes. A non-linear regression curve was obtained using the software Sigma plot 12.0 and the formula $Y = B_{MAX} X / [Kd + X]$, where Xs are fluorescent intensity, maximum fluorescence intensity, and concentrations of aptamers, respectively.

Results

Amplification of Aptamers and Optimization of the Selection Process

In this study, the asymmetric PCR was used to amplify a 78 nt-nucleotide library with the binding affinity to A₁ RBC antigen. To determine the annealing temperature of PCR, four temperatures of 60, 61, 62, 63, and 64 °C were measured. Most of the amplification products formed clear bands in 78n ton gel electrophoresis at 62 °C. The primer F created a more reliable and better product. Hence, it was chosen as the dominant primer for APCR (Figure-1). A number of 30 cycles were considered for APCR amplification. Given the role of magnesium chloride in the activation of the APCR reaction and to optimize Cell-Selex

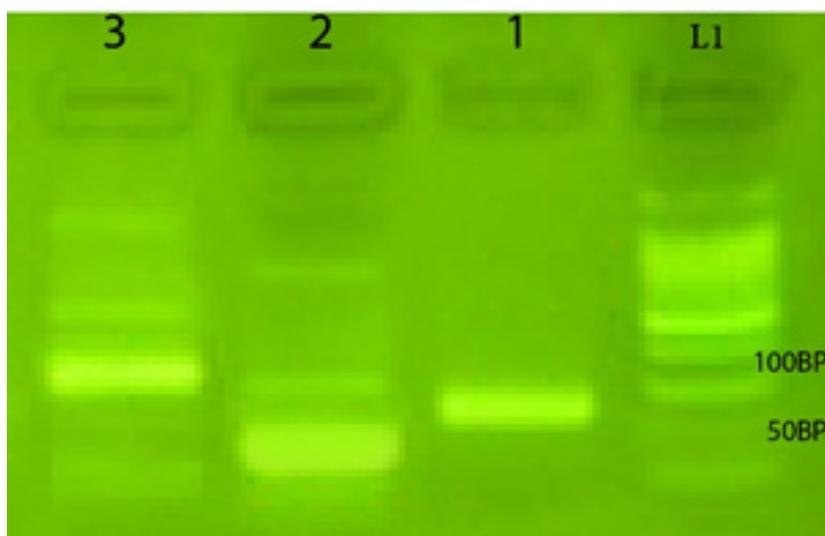


Figure 1. Gel electrophoresis of PCR products on agarose gel
L1: Ladder, **Row 1:** PCR product, **Row 2:** Asymmetric PCR product by primer F
Row 3: Asymmetric PCR product by primer R.

method during all stages of selection, the concentration of Mg was changed from 1 mm to 0.4 mm. Electrophoresis results on 2% agarose gel are exhibited in Figure-2.

Cell-Selex

Aptamers with a specific affinity to A₁ RBC antigen were selected following the 10th stage of the Cell-Selex process (Figure-2). Using Counter Selex, aptamers obtained from the 7th, 5th, and 3rd cycles were incubated with the RBC cells, the A₁ antigen of which was masked with A₁ polyclonal antibody in order to remove non-specific aptamers showing

affinities to target molecules. Then, the cells were precipitated, and the supernatant containing unattached aptamers was amplified and used in subsequent stages of the selection process. The enrichment and developed aptamers with affinity to A₁ RBC antigen were measured by fluorescence intensity of aptamers incubated in the cells at the 10th, 9th, 8th, 6th, and 4th cycles. Flow cytometry analysis yielded results of 3.3% in cycle 4 and 5.59% in cycle 6 representing a 69 percent increase in the specificity of aptamers to RBC compared to the previous steps (Figure-3). The results of the 8th, 9th, and 10th cycles were equal to 6.51%, 7.01%, and 7.04%, respectively, with

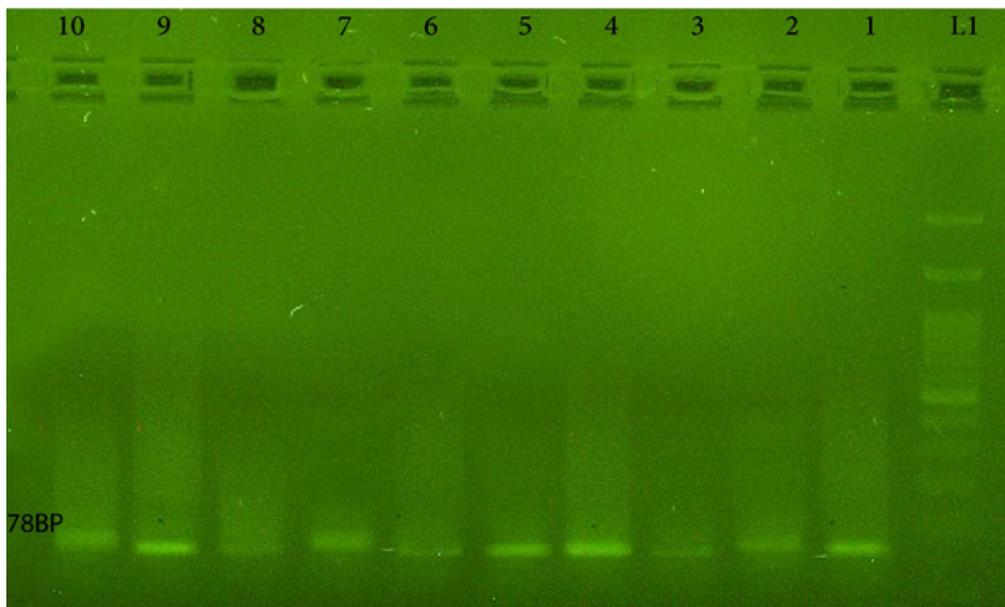


Figure 2. Gel electrophoresis of PCR product from the Selex selection process. L1: Ladder, and rows 1 to 10: PCR products' selection process of one to ten cycles.

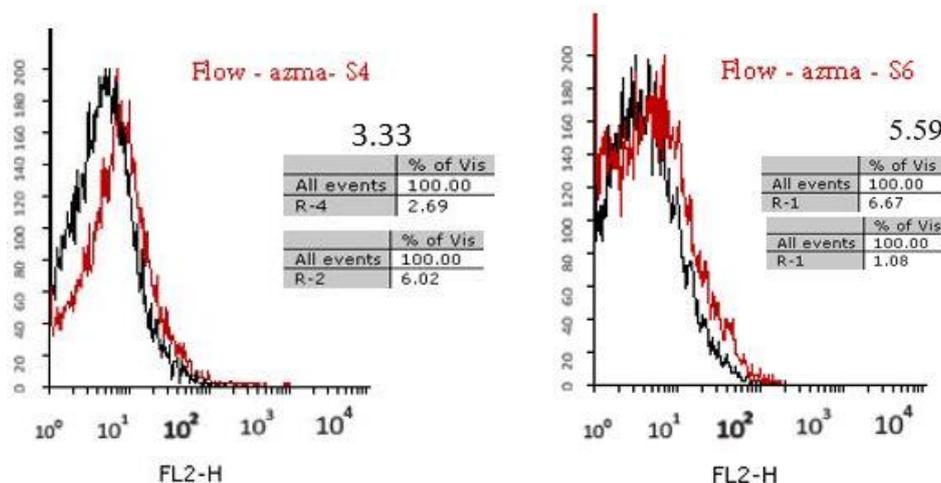


Figure 3. Percentage binding in Selex 4 and Selex 6.

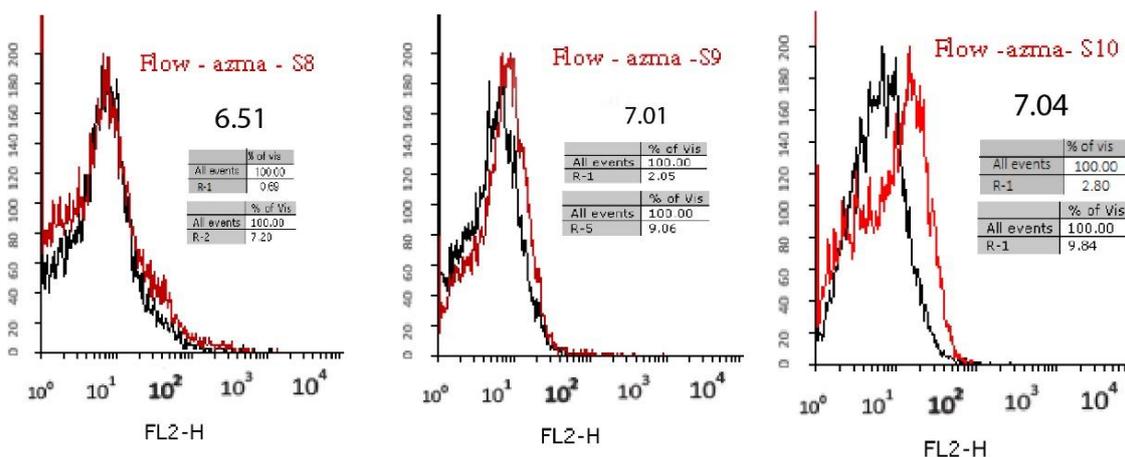


Figure 4. Percentage binding in Selex 8, Selex 9, and Selex 10

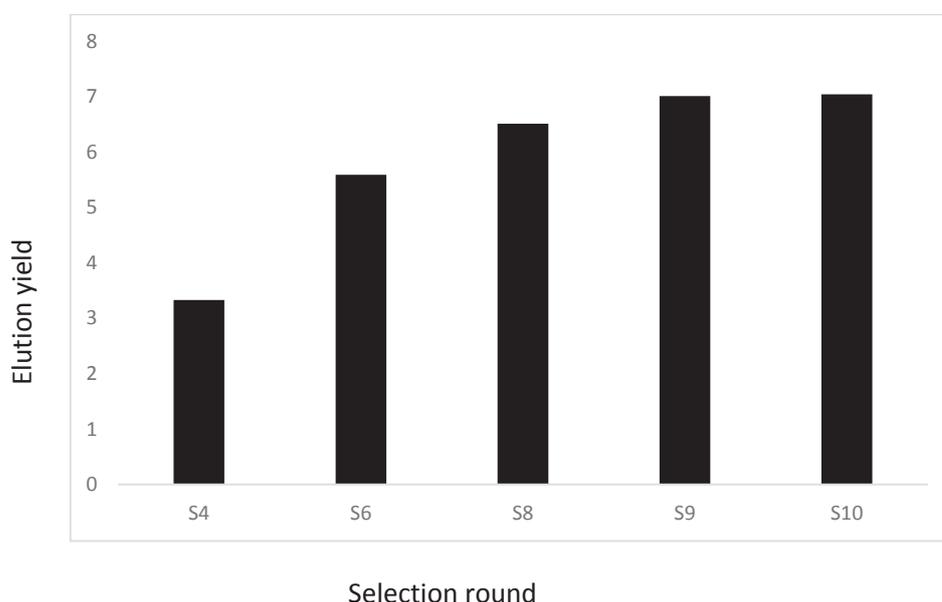


Figure 5. Comparing the fluorescent intensity of the selection process of Selex rounds of 4, 6, 8, 9, and 10

increased affinity specificity of 16%, 8%, and 0.4% (Figure-4 and 5). The Selex process was deemed to be terminated with partial equality of the results of the 10th and 9th cycles. Moreover, the results of Counter Selex steps yielded results of 4.37%, 1.05%, and 0.7% for the 3rd, 5th, and 7th cycles, respectively, with the binding affinities to the masked cells equalling -35% and -75% in the 5th and 7th cycles, respectively (Figure-6).

Cloning and Sequencing of Enriched Aptamer
The enriched aptamer library at the tenth stage of Selex was cloned with the highest binding affinity to A₁ RBC. The positive colonies were selected as a result of the transformation. Of the 38 aptamers transformed,

23 clones were confirmed by the PCR-colony as positive clones. The binding intensity, analyzed by flow cytometry of four random positive colonies, namely C₂, C₄, C₆, and C₈ was equal to 0.18, 1.84, 1.01, and 0.44%, respectively. Thus, C₄ colony with the highest intensity of fluorescence was selected and sequenced as an aptamer of choice based on the fluorescence intensity in A₁ RBC. The effectiveness of binding affinity to the cell for the final selected aptamer compared to the clone C₆ aptamer was assessed by flow cytometry. For this purpose, it was measured at a constant concentration of cells (2 x 10⁶) with different concentrations (0, 10, 50, 100, 200, and 300 Pmol) of aptamer labeled with FITC in a volume of 200 ml in binding buffer. The Kd

values of C₄ and C₆ clones were assessed at 29.5 ± 4.3 Pmol/L and 32.1 ± 2.7 Pmol/L, respectively (Figure-7).

Determining the Secondary Structure of the Aptamer

The secondary structure of the DNA aptamer was calculated using the basic software, mfold, based on free energy. Minimal energy (ΔG) of -13.13 kcal/mol was calculated for the stable structure of C₄ clone accordingly, and C₄₋₁ structure was predicted as the most stable structure of the aptamer for A₁ antigen (Figure-8). As shown in Figure 3–8, two secondary structures have been predicted for aptamer C₄, the free energy of which are ΔG=-13.13 kcal/mol for C₄₋₁ and ΔG=-12.88 kcal/mol for C₄₋₂. According to ΔG values, the structure with the lowest ΔG value was chosen as the best structure.

Discussion

Nowadays, aptamers are known as a new, capable, and flexible diagnostic tools as well as a convenient alternative to monoclonal antibody technology, which has been designed and produced for a lot of targets including the surfaces of human cells and pathogens [9]. Aptamers are single-strand RNA or DNA oligonucleotides that can identify a variety of target molecules by secondary structures. They are considered an important alternative due to a comparative advantage in the cost of production, ease of use, resistibility, and persistence compared to monoclonal antibodies [18]. Considering that 80 percent of people have blood group A of A₁ type [1], this study made an attempt to design a diagnostic aptamer for A₁ antigen using Cell-Selext method for single-stranded DNA from an oligonucleotide

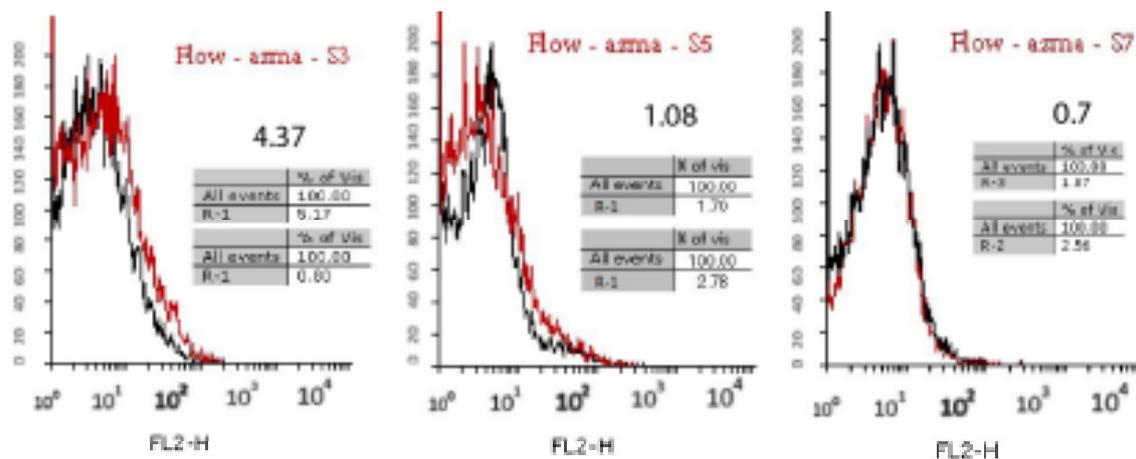


Figure 6. Comparing the fluorescent intensity of selection processes 3, 5, and 7 on the negative control cells

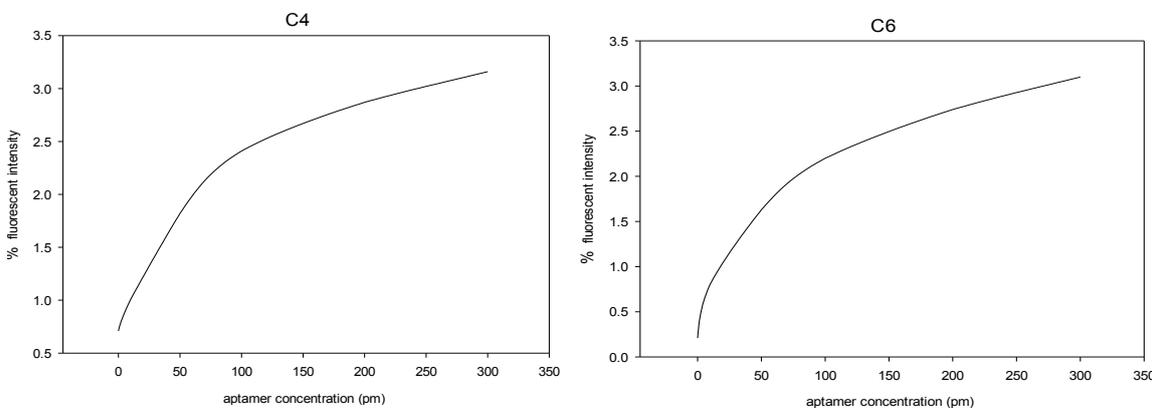


Figure 7. Non-linear regression curve for C4 kd clone (left) and C6 (right) to determine the binding affinity of various concentrations of aptamers C4 and C6 in relation to the cell constant.

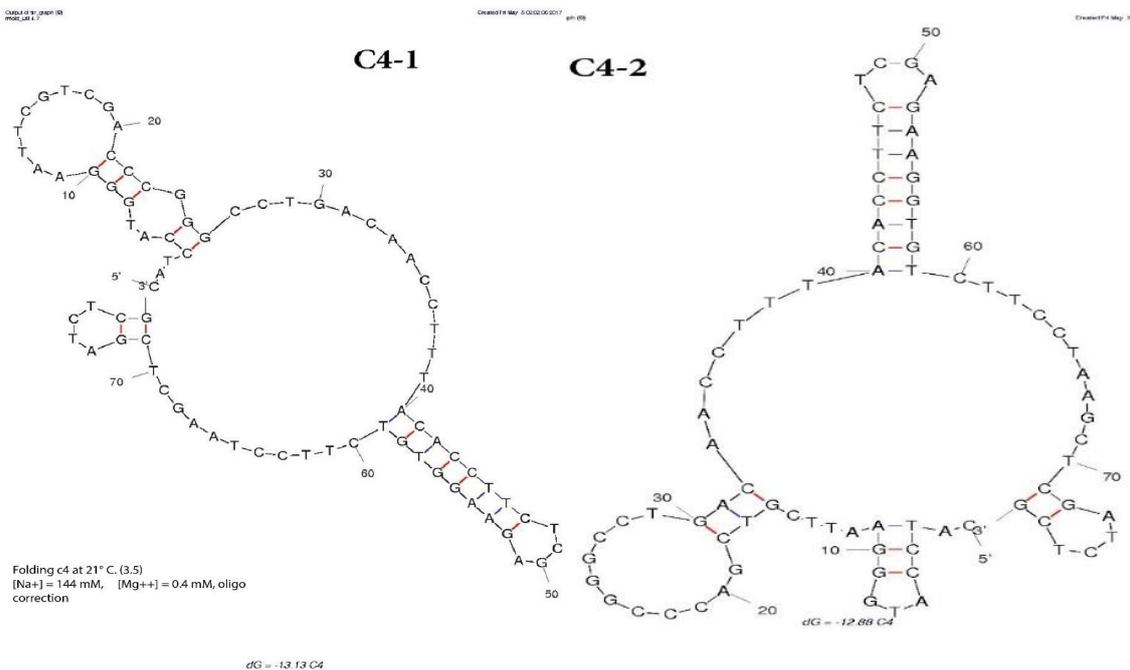


Figure 8. Prediction of two secondary structures for aptamer C4 based on mfold software.

pool that can replace antibodies. Cell-Selex has a special advantage for creating a specific and sensitive diagnostic tool based on the detection of cell surface antigens [19]. The aptamer designed in this study was of 78-mer nucleotides in which the number of random nucleotides and the middle was 38 nucleotides. PCR was used for the amplification of double-stranded DNA resulting from each stage of the selection process. Owing to the comparative advantage of asymmetric PCR, i.e. low cost and ease of use in ssDNA production unlike other methods, the dsDNA fragments were amplified and converted into ssDNA by APCR [20]. The thermal conditions and concentration of magnesium chloride enzyme were optimized. A temperature of 62 °C was chosen for amplification. The magnesium chloride concentration of 0.4 units per ml resulted in the highest production of dsDNA. A number of 30 cycles of PCR was considered for appropriate amplification [21]. Counter Selex was used for the reduction and elimination of non-specific sequences from the masked RBCs with A₁ polyclonal antibody during the selection process. According to the results of flow cytometry analysis of binding assays and progress of the selection process for aptamers achieved in the 4th, 6th, 8th, 9th, and 10th cycles labeled with FITC,

no considerable differences were found in the 8th, 9th, and 10th cycles compared with those from the initial library. Given that there is a high difference in fluorescence intensity between the final Selex and the synthetic initial library, the selection process was deemed to be completed in 10 stages. The intensity of fluorescence in negative control cells was reduced substantially compared to the initial library. After cloning of aptamers from the 10th selection stage, four clones were analyzed by fluorescence intensity, of which the cloned C₄ (1.84%) showed the highest affinity to A₁ RBC antigen. The dissociation constant of the aptamer against other targets was clearly in nano-molar to pico molar ranges [14, 18]. For example, the cloned aptamers to mosquito's RBC was reported to have a dissociation constant of 14 Mm to 84 Nm [22]. The results of binding examinations in this study revealed a C₄ equilibrium dissociation constant of 29.5±4.3 Pmol/L compared to the C₆. Hence, C₄ aptamer was selected as the final one. The stem and loop structures, as well as a low ΔG, are the key to the binding domain and the binding site in secondary structures of aptamer [18, 23]. Of the structures obtained, C₄₋₁ aptamer contains G4 and guanine-rich motifs. G4 motif can help a stable Scaffold aptamer. Of the secondary structures obtained for C₄

aptamer, two structures were attained by the web-based mfold software via a minimum energy level at 21°C and a sodium concentration of 114 Mm. The structure of C₄₋₁ ($\Delta G = -13.13$) was proposed as the best final structure. A specific aptamer was isolated for ARBC antigen in this study. The aptamer can detect A₁ RBC antigen in the ABO blood group system. Ni *et al.* used white blood cells for Counter Selex [24]. White blood cells, antigen B, and other blood group antigens can be used as Counter Selex to ensure the removal of non-specific targets and non-specific aptamer binding.

Conclusion

The aptamer designed has many advantages over A₁ monoclonal antibody in the detection

of blood group system that can replace antibodies in immunological diagnostic tests. It can also be used as part of a differential diagnostic tool such as biosensors and ELISA for the detection of the A₁ blood group.

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Conflict of Interest

The authors declare that there is no conflict of interest.

References

1. Procter J, Crawford J, et al. A rapid molecular method (polymerase chain reaction with sequence-specific primers) to genotype for ABO blood group and secretor status and its potential for organ transplants. *Tissue Antigens*. 1997; 50(5): 475-83.
2. Hosoi E. Biological and clinical aspects of ABO blood group system. *J Med Invest*. 2008;55(3-4): 174-82.
3. Breimer ME, Molne J, et al. Blood group A and B antigen expression in human kidneys correlated to A1/A2/B, Lewis, and secretor status. *Transplantation*. 2006;82(4): 479-85.
4. Malomgre W, Neumeister B. Recent and future trends in blood group typing. *Anal Bioanal Chem*. 2009;393(5): 1443-51.
5. Mascini M. Aptamers and their applications. *Anal Bioanal Chem*. 2008;390(4): 987-8.
6. Jayasena SD. Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clin Chem*. 1999;45(9): 1628-50.
7. Toh SY, et al. Aptamers as a replacement for antibodies in enzyme-linked immunosorbent assay. *Biosens Bioelectron*. 2015;64: 392-403.
8. Sefah K, et al. Development of DNA aptamers using Cell-SELEX. *Nat Protoc*. 2010;5(6): 1169-85.
9. Citartan M, et al. Conditions optimized for the preparation of single-stranded DNA (ssDNA) employing lambda exonuclease digestion in generating DNA aptamer. *World Journal of Microbiology and Biotechnology*. 2011;27(5): 1167-73.
10. Bai Y, Feng F, et al. Aptamer/thrombin/ aptamer-AuNPs sandwich enhanced surface plasmon resonance sensor for the detection of subnanomolar thrombin. *Biosens Bioelectron*. 2013;47: 265-70.
11. Citartan M, et al. Asymmetric PCR for good quality ssDNA generation towards DNA aptamer production. *Sonklanakarin Journal of Science and Technology*. 2012;34(2): 125.
12. Birch CM, et al. Identification of malaria parasite-infected red blood cell surface aptamers by inertial microfluidic SELEX (I-SELEX). *Sci Rep*. 2015;5: 11347.
13. Nuo Duan, et al. Selection, identification and application of a DNA aptamer against *Listeria monocytogenes*. *Food Control*. 2013;33: 239-243.
14. Dwivedi HP, et al. Selection of DNA aptamers for capture and detection of *Salmonella Typhimurium* using a whole-cell SELEX approach in conjunction with cell sorting. *Appl Microbiol Biotechnol*. 2013;97(8): 3677-86.
15. Kazunori Ikebukuro, et al. Selection of DNA aptamer against prostate specific antigen using a genetic algorithm and application to sensing. *Biosensors and Bioelectronics*. 2010;26: 1386-1391.

16. Jenkins PV, O'Donnell JS. ABO blood group determines plasma von Willebrand factor levels: a biologic function after all? *Transfusion*. 2006;46(10): 1836-44.
17. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*. 2003;31(13): 3406-15.
18. Duan N, et al. In vitro selection of a DNA aptamer targeted against *Shigella dysenteriae*. *J Microbiol Methods*. 2013;94(3): 170-4.
19. Sun MQ, et al. DNA aptamer selection in vitro for determining ketamine by FluMag-SELEX. *Fa Yi Xue Za Zhi*. 2014;30(5): 346-9.
20. Vahidi H, et al. Challenges to Design and Develop of DNA Aptamers for Protein Targets. II. Development of the Aptameric Affinity Ligands Specific to Human Plasma Coagulation Factor VIII Using SEC-SELEX. *Iran J Pharm Res*. 2017;16(2):737-44.
21. Nimjee SM, et al. Aptamers: an emerging class of therapeutics. *Annu Rev Med*. 2005;56: 555-83.
22. Meirinho SG, et al. Development of an electrochemical RNA-aptasensor to detect human osteopontin. *Biosens Bioelectron*. 2015;71: 332-41.
23. D'Andrea D, et al. ABO Blood Group and Rhesus Factor Are Not Associated with Outcomes After Radical Cystectomy for Non-metastatic Urothelial Carcinoma of the Bladder. *Anticancer Res*. 2017;37(10): 5747-53.
24. Ni X, et al. Nucleic acid aptamers: clinical applications and promising new horizons. *Curr Med Chem*. 2011;18(27): 4206-14.