

## Subtractive SELEX using agar beads for screening DNA aptamers with specific affinity to HIV gp41 antigen (Postprint)

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### Abstract

**Objective** To obtain DNA aptamers with highly specific affinity to HIV gp41 antigen via SELEX screening for HIV detection. **Methods** Specific DNA aptamers targeting HIV gp41 antigen were screened from double-stranded DNA derived from a single-stranded DNA (ssDNA) library, utilizing agarose beads as the solid support matrix and HIV gp41 antigen as the target molecule, through SELEX technology coupled with real-time quantitative PCR. **Results** The secondary ssDNA library obtained after six rounds of screening was amplified by PCR to produce dsDNA. The dsDNA was ligated into the pMDTM18-T vector, followed by cloning and sequencing, which yielded four aptamers against HIV gp41 antigen. The binding affinities ( $K_d$ ) of all four aptamers reached the nanomolar range. Among them, aptamer No. 15 displayed the highest affinity. Specificity analysis revealed that all four aptamers exhibited specific binding to HIV gp41 antigen without cross-reactivity to other non-specific proteins. **Conclusion** We successfully isolated DNA aptamers with highly specific affinity for HIV gp41 antigen from a random single-stranded oligonucleotide library, and the obtained aptamers possess antagonistic activity against HIV gp41 antigen.

### Full Text

#### Preamble

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#### Original Article

#### Subtractive SELEX Using Agar Beads for Screening DNA Aptamers with Specific Affinity to HIV gp41 Antigen

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## Abstract

**Objective:** To obtain DNA aptamers with highly specific affinity to HIV gp41 antigen through SELEX screening for HIV detection.

**Methods:** Specific DNA aptamers against HIV gp41 antigen were screened from a single-stranded DNA (ssDNA) library using agarose beads as the solid support, HIV gp41 antigen as the target molecule, SELEX technology, and real-time quantitative PCR.

**Results:** After six rounds of screening, the secondary ssDNA library was amplified by PCR to obtain double-stranded DNA (dsDNA). The dsDNA was ligated into pMDTM 18-T vector, cloned, and sequenced, yielding four aptamers against HIV gp41 antigen. The binding affinities (Kd) of all four aptamers reached the nanomolar level, with aptamer No.15 showing the strongest affinity. Specificity analysis revealed that all four aptamers bound specifically to HIV gp41 antigen without detectable affinity for non-specific proteins.

**Conclusion:** We successfully obtained DNA aptamers with highly specific affinity to HIV gp41 antigen from a random single-stranded oligonucleotide library, and the selected aptamers demonstrated the ability to antagonize HIV gp41 antigen.

**Keywords:** HIV gp41 antigen; subtractive SELEX technique; DNA aptamers

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## Introduction

HIV infection stimulates the production of antibodies against viral antigens such as envelope proteins (gp120 and gp41) and core protein (p24). Typically, HIV can be detected two to three weeks post-infection, and viral antigens in blood serve as serum markers during the AIDS window period, enabling early diagnosis and treatment. Currently, the two main strategies for antigen recognition and detection rely on antibody-antigen interactions and DNA aptamer-antigen binding. Compared with antibodies, DNA aptamers offer numerous advantages including higher stability due to their low degradation rate, stronger and more specific binding affinity to target molecules, shorter screening cycles (one to two months), low immunogenicity, potential for repeated application due to rapid denaturation and renaturation, absence of effector functions, capability for chemical modification, and lower cost.

To date, various DNA aptamers with specific binding affinity to gp120 and p24 have been screened for antigen detection, demonstrating better sensitivity

and facile application. However, no DNA aptamer with highly specific affinity to gp41 antigen—which plays an important role in viral packaging and maturation—has been reported. Herein, we report for the first time the screening of DNA aptamers with specific binding affinity to gp41 antigen through subtractive SELEX screening using commercial carboxyl-enriched agar magnetic beads as the separation medium and subsequent conventional quantitative electrophoresis. These aptamers provide a basis for efficient, rapid, and accurate detection of gp41 antigen and may greatly facilitate the development of future high-throughput HIV screening methods with a clear molecular mechanism.

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## Materials and Methods

### Materials

The single-stranded DNA (ssDNA) library (5'-CTATAGCAATGTCAAAGTGCACGCTACTTGTACGGTAC-3'), P6 primer (5'-TGCTAATTTAGCCAATGGTACGGTACTTCC-3'), universal primer M13-47 (5'-TCACGACT-3'), P9 primer (5'-CGCCAGGGTTTCCAG-3'), biotin-labeled primers, and PCR mix were purchased from TaKaRa. pMDTM 18-T vector and Gel Extraction Kit were obtained from KGI Nanjing Biological Technology Development Co., Ltd.

Buffer solutions: PBS (0.01 mol/L, pH 7.4) was purchased from Jiangsu Bollywood Biotechnology Co., Ltd, containing 137 mmol/L NaCl, 2.7 mmol/L KCl, 6.5 mmol/L Na HPO<sub>4</sub>, 1.8 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 2.5 mmol/L MgCl<sub>2</sub>, 1 mmol/L CaCl<sub>2</sub>, and 1% (mass ratio) bovine serum albumin (BSA), used as 1× screening buffer. The 1× binding buffer contained PBS (0.01 mol/L, pH 7.4), 1 mmol/L CaCl<sub>2</sub>, and 1 mg/mL BSA. The blocking solution contained 0.01 mol/L PBS, 1 mg/mL BSA, and 1 mg/mL salmon sperm DNA.

### Aptamer Selection

**ssDNA Library Pretreatment:** The ssDNA library was centrifuged at 10,000 r/min for 5 min, followed by addition of 600  $\mu$ L binding buffer and thorough mixing.

**Two Rounds of Positive Screening with HIV gp41 Antigen:** In the first round, 0.3 mL magnetic beads and 300  $\mu$ L HIV gp41 antigen (diluted according to instructions) were added to a 1.5 mL EP tube and incubated for 2 h at 37°C. The product was magnetically separated and rinsed three times with screening buffer, followed by addition of 500  $\mu$ L blocking solution. After incubation at 37°C for 1 h, the product was separated and washed twice with screening buffer (1 min each) and once with binding buffer for 1 min before adding the primary ssDNA library. The mixture was treated at 95°C for 5 min, 4°C for 10 min, and 37°C for 5 min, then incubated for 1 h at 37°C. The magnetic bead-ssDNA library complexes were separated and washed three times with screening buffer (1 min each). Deionized water (200  $\mu$ L) was added and the mixture was heated

at 95°C for 10 min. The supernatant was collected as the secondary library for the next screening round.

The second round followed identical procedures except the magnetic bead volume was 0.15 mL.

**Amplification of ssDNA to dsDNA by qPCR:** PCR template (160 L) was added to half of the above solution and mixed thoroughly. The homogeneous mixture was divided into 8 equal portions (30 L each) for qPCR until the amplification fluorescence curve reached its highest point.

**Preparation of Secondary ssDNA Library:** Streptavidin-biotin magnetic beads (0.1 mL) and amplified double-stranded DNA (dsDNA) were added to a 1.5 mL EP tube and incubated for 30 min at 37°C. The recovered beads were incubated with 500 L of 1% (mass ratio) TPBS at 37°C for 4 min, repeated three times. The beads were washed with water, followed by addition of 200 L binding buffer. After incubation at 95°C for 5 min, the recovered supernatant was added to a counter-screening tube and incubated at 37°C for 45 min. The recovered supernatant was subsequently added to a positive screening tube and incubated for 1 h to remove unbound ssDNA library. All recovered beads after positive screening were washed three times with screening buffer (3 min each). Deionized water (200 L) was added, and after incubation at 95°C for 5 min, the supernatant was recovered as secondary libraries for the next screening round.

**Enrichment Effect Monitoring:** PCR template (10 L) was added to 20 L of recovered supernatant after positive screening and counter-screening, respectively. Solutions were amplified by qPCR, with 20 L PCR template in 10 L deionized water as the control sample.

**SELEX Screening with HIV P24 Antigen as Counter-Screening Target:** SELEX screening with HIV P24 antigen as the counter-screening target molecule was conducted following identical procedures described above except gp41 antibody was replaced by HIV P24 antigen in the third screening round. Conditions for each SELEX round are listed in .

### Cloning and Sequencing of Selected Aptamers

The dsDNA obtained from the positive screening tube after six selection rounds was used as template to prepare a 300 L PCR reaction system containing 30 L of 10× buffer with Mg<sup>2+</sup>, 45 L template, 2.4 L dNTP, 3 L P6 primer, 3 L P9 primer, 2 L rTaq enzyme, and 214.6 L deionized water. The homogeneous PCR reaction was divided into 6 equal portions and amplified by routine PCR for 26 thermal cycles (95°C for 5 min, 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s). The dsDNA fragment was recovered from the thermally cycled solution via gel electrophoresis.

**Connection of dsDNA Fragment with pMDTM18-T Vector:** The recovered dsDNA was dissolved in deionized water and ligated with pMDTM18-T vector to prepare the connection solution containing 0.5 L T-vector, 2 L insert

fragment, 5  $\mu$ L solution, and 383.5  $\mu$ L deionized water. The connection solution was incubated at 16°C for 30 min to obtain the secondary library dsDNA recombinant plasmid.

**E. coli Transformation and Selection of Positive Clones:** E. coli DH5 competent cells were prepared by treatment with 0.1 mol/L CaCl<sub>2</sub> and stored at -80°C. For transformation, 10  $\mu$ L recombinant DNA or 10  $\mu$ L deionized water was added to thawed competent cells, which were plated on LB solid medium containing ampicillin to select positive clones.

The dissociation constant (K<sub>d</sub>) for aptamer-HIV gp41 antigen interaction was determined using the equation. The aptamer selection process by SELEX is illustrated in [Figure 1: see original paper].

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## Results

### Staggered Extension Process (StEP) for Identifying Positive Clones

Monoclonal bacteria preserved at -80°C were used as template with a blank control group. The staggered PCR system using universal primers M13-47 and P6/P9 consisted of 10  $\mu$ L 10 $\times$  PCR buffer with Mg<sup>2+</sup>, 2  $\mu$ L template, 8  $\mu$ L dNTP, 1  $\mu$ L primer P6/P9, 1.5  $\mu$ L rTaq enzyme, and 80.7  $\mu$ L deionized water. The homogeneous PCR reaction was divided into 10 equal portions and amplified by routine PCR for 30 thermal cycles (95°C for 5 min, 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s). The plasmid insertion direction was determined by non-denaturing polyacrylamide gel electrophoresis.

### Identification of Specific Affinity of HIV gp41 Antigen Aptamers to Positive Clones

Positive clones identified by StEP were used as templates to synthesize ssDNA via asymmetric PCR. Agar beads (0.2 mL) were packed in four tubes labeled with HIV gp41 antigen, anti-HBsAg antibody, HIV P24 antigen, and BSA, respectively. Specific aptamer affinities to these proteins were determined by qPCR analysis.

### Flow Cytometry Analysis

The specific affinities of the four aptamers to HIV gp41 antigen were measured to identify the aptamer with strongest specificity. To monitor aptamer enrichment after selection, beads binding to HIV gp41 antigen ( $1 \times 10^6$ ) and beads binding to HIV P24 antigen ( $1 \times 10^6$ ) were incubated with FITC-labeled ssDNA ( $f = 200$  nmol/L) in binding buffer ( $V_f = 200$   $\mu$ L) at 37°C for 45 min. Fluorescence intensity was monitored using a FACS Aria cytometer by counting 10,000 events. The unselected FITC-labeled ssDNA library served as negative control.

Aptamer binding affinities were evaluated by flow cytometry, with the initial FITC-labeled library as negative control to determine background. All binding assays were performed in triplicate.

### **Selection of DNA Aptamers Against HIV gp41 Antigen**

Through two rounds of positive screening and four rounds of counter-screening, four highly specific aptamers against HIV gp41 antigen were obtained. qPCR results from the third round of positive selection ([Figure 2: see original paper]) showed enrichment of selected aptamers in the positive selection tube. Although specific aptamers binding the fixed target gradually increased in the ssDNA library, enrichment did not reach saturation, indicating that further screening with increased selection pressure was needed to obtain highly specific aptamers binding HIV gp41 antigen.

As shown in [Figure 3: see original paper], qPCR results from the fourth and fifth rounds of counter-selection demonstrated that specific aptamers binding HIV gp41 antigen reached saturation in the positive selection tube, while non-specific ssDNA could be rapidly eliminated by magnetic bead-based screening to increase enrichment of specific aptamers.

### **Sequencing Results and Secondary Structures of Aptamers**

Products from the sixth screening round were cloned and sequenced to obtain the sequences of four aptamers (). The secondary structures of the HIV gp41 antigen aptamers are shown in [Figure 4: see original paper]. [Figure 5: see original paper] shows enrichment of DNA sequences binding to HIV P24 and gp41 antigens.

### **Positive Clones Identified by StEP Technique**

Positive clones were identified by StEP technique. A clear combined band of 170-180 bp appeared in some clones but not others, demonstrating successful transformation by recombinant plasmids. The primer combination M13-47 and P7x producing nucleic acid bands at 170-180 bp indicated forward insertion of the fragment into the vector, while the combination M13-47 and P11x with bands at 170-180 bp indicated reverse insertion; no such bands appeared in the blank group. A total of nine positive monoclonal clones were identified, including four with forward insertion and five with reverse insertion ([Figure 6: see original paper]).

### **Specific Binding Between HIV gp41 Antigen Aptamer and Positive Clones**

[Figure 7: see original paper] shows successful conversion of positive clones with specific binding to HIV gp41. The No.15 positive clone exhibited highly specific affinity to HIV gp41 without adsorption to other non-specific proteins.

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## Discussion

The SELEX technique requires designing and synthesizing random ssDNA libraries of suitable length (60-100 bp). The central segment contains 20-60 random base pairs flanked by primer annealing sequences. Theoretically, a random sequence of 20 bases can form at least  $1 \times 10^{12}$  different sequences, providing a library storage capacity of at least  $1 \times 10^{13}$ .

Magnetic beads serve as an excellent separation medium due to their affinity for various target proteins. Bound ligands can be rapidly separated from unbound ssDNA library by washing with screening buffer. Since biotin-streptavidin interaction is the strongest known in nature, commercial streptavidin-biotin magnetic beads were utilized to screen HIV gp41 antigen aptamers.

Library enrichment is a critical indicator of SELEX screening success. Aptamers with affinity to HIV gp41 antigen were successfully obtained after multiple rounds of screening and counter-screening. qPCR analysis of positive screening in the third round of counter-selection showed that screened aptamers began enriching in the positive selection tube. Although specific aptamers binding the fixed target gradually increased in the ssDNA library, enrichment did not reach saturation. After the fourth round of counter-selection, specific aptamers binding HIV gp41 antigen reached saturation in the selection tube. Subsequent StEP analysis and Kd value measurement indicated that aptamers obtained by magnetic bead-based SELEX possessed high specificity to HIV gp41 antigen. The utilization of qPCR, gel electrophoresis, and other conventional techniques simplifies aptamer screening procedures and shortens screening time. The obtained aptamers may facilitate future high-throughput screening and development of rapid, low-cost HIV test kits.

In this study, aptamers with highly specific affinity to HIV gp41 antigen were successfully obtained after six rounds of screening with magnetic beads. To minimize non-specific ssDNA adsorption and obtain highly specific aptamers, coupled targets in the counter-selection tube were replaced in the third screening round. The specific aptamer obtained provides an efficient, rapid, and accurate probe for early HIV diagnosis through gp41 antigen detection.

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