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 a highly specific affinity to HIV gp41 antigen using SELEX screening for detection of HIV. **Methods** The specific DNA aptamers of HIV gp41 antigen were screened from the double-stranded DNA derived from the single-stranded DNA (ssDNA) library with agarose beads as the supportive medium and HIV gp41 antigen as the target molecule using SELEX technique and real-time quantitative PCR. **Results** The secondary ssDNA library obtained after 6 rounds of screening was amplified by PCR to obtain dsDNA. The dsDNA was linked with pMD™ 18-T vector, cloned and sequenced to obtain 4 aptamers of HIV gp41 antigen. The affinities of the 4 aptamers (K_d) all reached the nanomolar level. Among the 4 aptamers, the No.15 aptamer showed the strongest affinity. Specificity analysis of the aptamers revealed that all these 4 aptamers had specific affinity to HIV gp41 antigen with no affinity to other non-specific proteins. **Conclusion** We successfully obtained DNA aptamers with highly specific affinity to the HIV gp41 antigen from random single-stranded oligonucleotide library, and the obtained aptamers have the ability to antagonize HIV gp41 antigen.

Key words: HIV gp41 antigen; subtractive SELEX technique; DNA aptamers

INTRODUCTION

HIV infection is known to stimulate the production of antibodies against such antigens as envelope proteins (gp120 and gp41) and core protein (P24) of HIV [1, 2]. Typically, HIV can be detected two or three weeks after its infection, and the antigens in the blood can serve as serum markers during the window period of AIDS to allow early diagnosis and treatment [3, 4]. Currently the two main strategies for recognition and detection of the antigens rely on the detection of the interactions between antibody and antigen and between DNA aptamers and antigen [5]. Compared with antibody/antigen interaction, DNA aptamers have many advantages including a higher stability due to their low degradation rate [6, 7], a stronger and specific binding affinity to the target molecule [8, 9], a shorter screening cycle (one or two months) [10, 11], a low immunogenicity, potential of repeatable application due to their quick denaturation and renaturation, absence of the function of effector, a better sensitivity and facile chemical modification [12, 13], and a lower cost. So far a variety of DNA aptamers with specific binding affinity to gp 120 and P24 have been screened for detecting the antigens for early diagnosis of HIV infection [14, 15], but no such a DNA aptamer has been reported with a highly specific affinity to the antigen gp 41, which plays an important role in the process of virus packaging and maturation of HIV [16, 17].

Herein we report, for the first time, the screening of 4 DNA aptamers with specific binding affinity to gp41 antigen through subtractive SELEX screening with commercial carboxyl-enriched agar magnetic beads as the separation medium and subsequent conventional quantitative PCR (qPCR) technique and gel electrophoresis. These aptamers provide a basis for efficient, rapid and accurate detection of gp41 antigen for early diagnosis of HIV [18, 19] and may greatly facilitate the development of future high-throughput screening of HIV with a clear molecular mechanism [20].

MATERIALS AND METHODS

Materials

The single-stranded DNA (ssDNA; 5'-CTATAGCAATG GTACGGTACTTCC[40N]-CAAAAGTGCACGCTACTT-TGCTAA-3'), P6 primer (5'-TAATACGACTCACTATAG CAATGGTACGGTACTTCC-3'), P9 primer (5'-TTAGC AAAGTAGCGTGCACTTTTG-3'), biotin-P9 primer, universal primer M13-47 (5'-CGCCAGGGTTTTCCCAG TCACGAC-3'), and PCR mix were purchased from TaKaRa. pMD™ 18-Tvector vector and the Gel Extraction

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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, which contained 137 mmol/L of NaCl, 2.7 mmol/L of KCl, 6.5 mmol/L of Na_2HPO_4, 1.8 mmol/L of NaH_2PO_4, 2.5 mmol/L of $MgCl_2$, 1 mmol/L of $CaCl_2$, and 1% (mass ratio) bovine serum albumin (BSA) and used as the 1×screening buffer. The 1×binding buffer contained PBS (0.01 mol/L, pH 7.4), 1 mmol/L of $CaCl_2$, and 1 mg/mL of BSA. The blocking solution contained 0.01 mol/L of PBS, 1 mg/mL of BSA, and 1 mg/mL of salmon sperm DNA.

Aptamer selection

ssDNA library pretreatment ssDNA library was centrifuged at 10 000 r/min for 5 min followed by addition of 600 μL binding buffer, and the mixture was well mixed.

Two rounds of positive screening with HIV gp41 antigen In the first round of positive screening, 0.3 mL of magnetic beads and 300 μL of HIV gp41 antigen (diluted at 1∶100 according to the instruction) were added in a 1.5-mL EP tube and incubated for 2 h at 37 ℃. The product was magnetically separated and rinsed with the screening buffer for 3 times, followed by the addition of 500 μL of the blocking solution. After incubation at 37 ℃ for 1 h, the product was separated and washed with the screening buffer twice (for 1 min each time) and with the binding buffer once for 1 min before the addition of the primary ssDNA library. The above mixture was treated at 95 ℃ for 5 min, 4 ℃ for 10 min, and 37 ℃ for 5 min, and then incubated for 1 h at 37 ℃. The composites of magnetic beads and ssDNA library were separated and washed for 3 times with the screening buffer (1 min each time); 200 μL deionized water was then added and the mixture was centrifuged at 95 ℃ for 10 min. The supernatant was collected for use as the secondary library for the next round of screening. The second round of positive screening was carried out following the identical procedure in the first round of screening except that the amount of magnetic beads was 0.15 mL.

Amplification of ssDNA into dsDNA by qPCR The PCR template (160 μL) was added in half of the above solution and mixed thoroughly. The homogeneous mixture was then divided into 8 equal portions (30 μL) for qPCR until the amplification fluorescence reached the highest point of the S-curve.

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

Enrichment effect monitoring PCR template (10 μL) was added into 20 μL of recovered supernatant after positive-screening and counter-screening, respectively. The corresponding homogeneous solutions were amplified by qPCR, with 20 μL of PCR template in 10 μL of deionized water as the control sample.

SELEX screening with HIV P24 antigen as counter-screening target molecule SELEX screening with HIV P24 antigen as the counter-screening target molecule was conducted following identical screening procedures described above except for the replacement of gp41 antibody by HIV P24 antigen in the third round of screening. The conditions in each round of SELEX screening were listed in Tab.1.

Cloning and sequencing of selected aptamers

dsDNA obtained through secondary library The recovered supernatant in the positive-screening tube after 6 rounds of selection was used as the template to form 300 μL of PCR reaction system, which consisted of 30 μL of 10 × buffer containing Mg^{2+}, 45 μL of template, 2.4 μL of dNTP, 3 μL of P6 primer, 3 μL of P9 primer, 2 μL of rTaq enzyme, and 214.6 μL of deionized water. The homogeneous PCR reaction was divided into 6 equal portions and amplified by routine PCR for 26 thermal cycles of 95 ℃ for 5 min, 94 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 30 s. The dsDNA fragment was recovered through gel electrophoresis from the thermally-cycled solution.

Connection of dsDNA fragment with pMD™18-T vector The recovered dsDNA was dissolved in deionized water and connected with pMD™18-T vector to prepare the connection solution, which consisted of 0.5 μL of pMD™18-Tvector, 2 μL of inserted fragment, 5 μL of solution and 383.5 μL of deionized water. The connection solution was reacted at 16 ℃ for 30 min to obtain the secondary library dsDNA recombinant plasmid.

E. coli transformation and selection of positive clones The original strain of *E. coli* DH5α was cultured and shaken till logarithmic growth (with absorbance at 600 nm of 0.6). Competent bacterial cells (200 μL) were prepared by treatment with 0.1 mol/L $CaCl_2$ and

Tab.1 Conditions of 6 rounds of SELEX screening

Round	Magnetic bead volume (mL)	DNA input (pmol)	Template RNA input (μmol/L)	Washing times	Bonding time (h)	HIV gp41 antigen binding efficiency
1	0.30	2500	10	3	1	15%
2	0.15	1000	10	3	1	-
3	0.15	300	10	4	1	32.6%
4	0.15	300	10	5	1	41.7%
5	0.15	300	10	5	1	52.9%
6	0.15	300	10	6	1	63%

transferred into a 1.5-mL EP tube for storage at −80 ℃ before use. For *E. coli* transformation, 10 μL of recombinant DNA or 10 μL of deionized water were added into the thawed competent cells, which were coated on the plate and cultured on a solid LB medium containing ampicillin to select the positive clones.

Stagger extension process (StEP) to identify positive clones The monoclonal bacteria preserved at −80 ℃ was used as a template system, and the blank group was set. Staggered PCR system of the universal primers M13-47 and P6/P9 consisted of 10 μL of 10 × PCR buffer containing Mg^{2+}, 2 μL of template, 8 μL of dNTP, 1 μL of primer P6/P9, 1.5 μL of rTaq enzyme and 80.7 μL of deionized water. The homogeneous PCR reaction was divided into 10 equal portions and amplified by routine PCR for 30 thermal cycles (95 ℃ for 5 min, 94 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ 30 s). The direction of the target fragment inserted into the plasmid was determined by non-denaturing polyacrylamide gel electrophoresis.

Identification of specific affinity of HIV gp41 antigen aptamer to positive clones The positive clones identified by StEP technique were used as the template to synthesize ssDNA using asymmetric PCR method. Agar beads (0.2 mL) were packed in 4 tubes labeled with "+", "1 -", "2 -", "3-", which were coupled with HIV gp41 antigen, anti-HBsAg antibodies, HIV P24 antigen, and BSA, respectively. The specific affinities of the aptamers to these proteins were determined with qPCR analysis.

Flow cytometry analysis The specific affinities of the four aptamers to HIV gp41 antigen were measured to identify the aptamer with the strongest specificity. To monitor the enrichment of the aptamers after selection, 1×10^5 beads binding to HIV gp41 antigen and 1×10^5 beads binding to HIV P24 antigen were incubated with FITC-labeled ssDNA in the binding buffer (C_l=200 nmol/L, V_l=200 μL) at 37 ℃ for 45 min. The fluorescence intensity of the beads was monitored with a FACS Aria cytometer by counting 10 000 events. The FITC-labeled unselected ssDNA library served as the negative control. The binding affinities of the aptamers were evaluated by flowing cytometry, with the initial FITC-labeled library as the negative control to determine the background binding. All the binding assays were performed 3 times. The K_d of the interaction between the aptamer and the HIV gp41 antigen was determined using the equation $Y=B_{max}X/(K_d+X)$.

The process of aptamer selection by SELEX was illustrated in Fig.1.

RESULTS

Selection of DNA aptamers against HIV gp41 antigen

Through two rounds of positive screening and 4 rounds of negative screening, 4 highly specific aptamers of HIV gp41 antigen were obtained. The qPCR results of positive selection and negative selection in the third-round counter-selection (Fig.2) showed enrichment of the selected aptamers in the positive-selection tube. Although the specific aptamers binding with the fixed target gradually increased in ssDNA library, aptamer enrichment did not reach saturation. Further screening with an increased screening pressure was needed to obtain the highly specific aptamers binding with HIV gp41 antigen.

As shown in Fig.3, qPCR results of positive selection and counter selection in the fourth round of counter selection showed that the enrichment of the specific aptamers binding with HIV gp41 antigen reached saturation in the positive-selection tube, suggesting that the non-specific ssDNA could be quickly eliminated by magnetic bead-based screening to increase the enrichment of the specific aptamers.

Sequencing result and secondary structures of the 4 aptamers

The products of the sixth round of screening were cloned and sequenced to obtain the sequences of the 4 aptamers (Tab.2). The secondary structures of the 4 aptamers of HIV gp41 antigen were shown in Fig.4. Fig.5 shows the enrichment of the DNA sequences that bind to HIV P24 and gp41 antigens.

Positive clones identified by StEP technique

The positive clones were identified by StEP technique. A clear combined band of 170-180 bp appeared in some clones but not in the other clones, demonstrating successful transformation by the recombinant plasmids. The selected combination of primers M13-47 and P7x

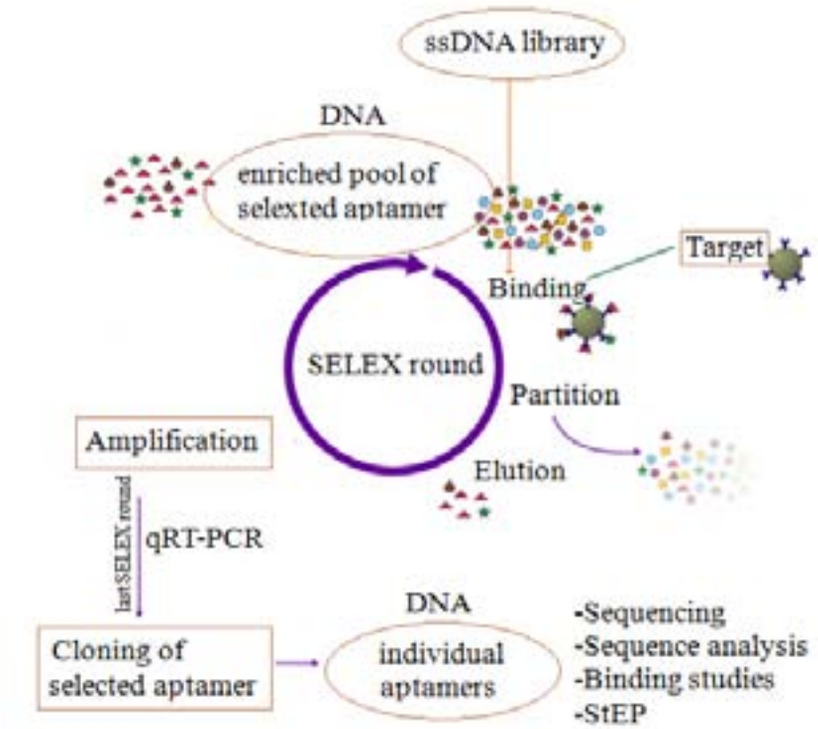

Fig.1 Process of aptamer selection by SELEX.

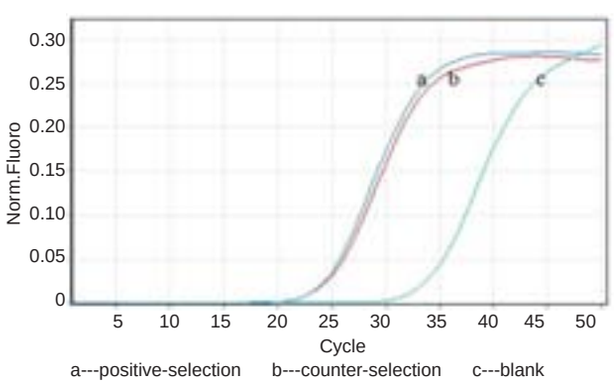

Fig.2 qPCR detection of the third round of negative selection.

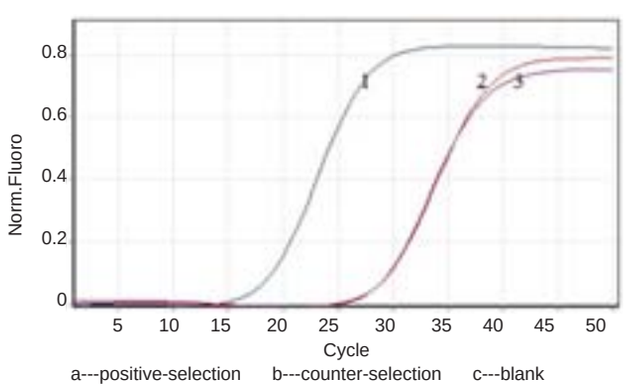

Fig.3 qPCR detection after the fourth and fifth rounds of counter-selection.

with nucleic acid bands at 170-180 bp demonstrated that the fragment was inserted forwardly into the vector, and the selected combination of the primers M13-47 and P11x with bands at 170-180 bp indicated a reverse insertion of the fragment in the vector; no such bands occurred in the blank group following amplification with the two primers. A total of 9 positive monoclones were identified, including 4 with positive insertion and 5 with reverse insertion as shown by electrophoresis analysis (Fig.6).

Specific binding between HIV gp41 antigen aptamer and positive clones

Fig.7 shows successful conversion of the positive clones with specific binding with HIV gp41. The No.15 positive clone had a highly specific affinity to HIV gp41 without adsorption to other non-specific proteins.

DISCUSSION

SELEX technique is the first requirement for designing and synthesizing random ssDNA libraries of suitable length (60-100 bp). The central segment of the library is a random sequence containing 20-60 base pairs flanked by a primer annealing sequence as the lateral segments. Theoretically, a random sequence contains 20 bases of random ssDNA library, in which at least 1×10^{15} different sequences could be formed so that the storage capacity of the library could reach at least 1×10^{15}.

Magnetic beads can be used as a good separation medium due to their affinity to a variety of target proteins. Ligands bound to the beads could be rapidly separated from unbound ssDNA library by washing the

Tab.2 Sequences of the 4 aptamers binding with HIV gp41 antigen

Name	Size (bp)	Sequence (5'-3')	K_d (nmol/L)
1	40	ATCGGATGGGGACTCACCAGCTGACAGCTTGCCCTTTAGC	98+7.2
7	40	CCCCTGCGTTGAATCGGGTCGCCACGCCTGGTTGGAAGGA	51+4.9
10	40	TCGGCACGTTCTCAGTAGCGCTCGCTGGTCATCCCACAGC	32+9.6
15	40	GGACGCGGTTAGGGGAGAATTTCCATACCTTTGGGTTGTA	26+6.6

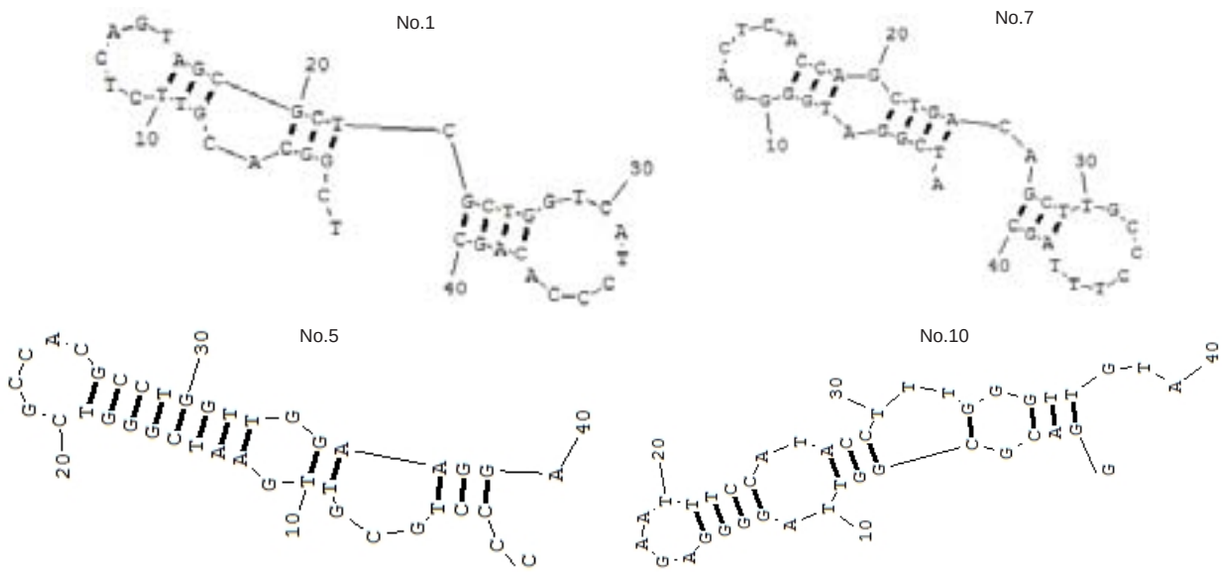

Fig.4 Stem-loop structures of the 4 selected aptamers binding with HIV gp41 antigen as predicted by RNA Structure 3.7 software.

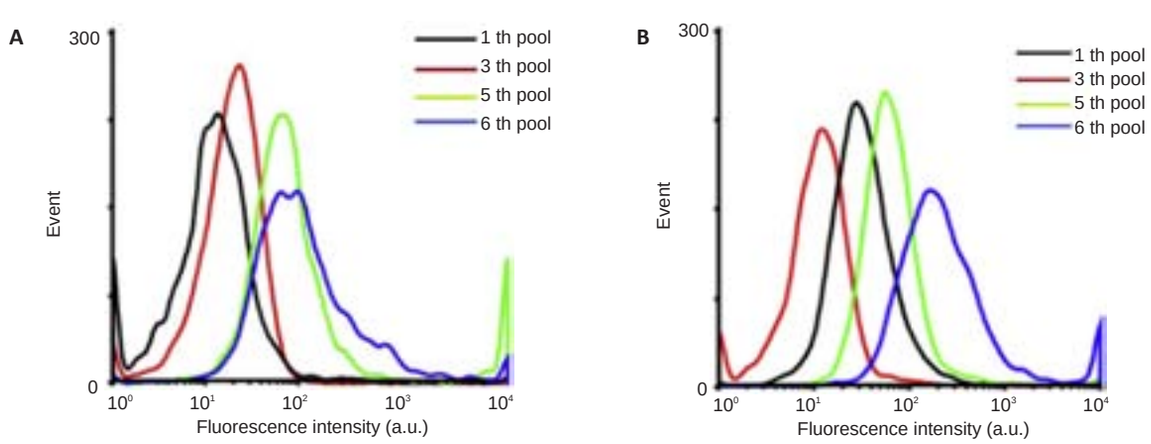

Fig.5 Flow cytometry to monitor the specific affinity of the beads to HIV antigens P24 (***A***) and gp41 (***B***).

beads with the screening buffer. As biotin and streptavidin have the strongest interaction known in nature, commercial streptavidin-biotin magnetic beads were utilized to screen HIV gp41 antigen aptamers.

The library enrichment is an important indicator of SELEX screening. The aptamers with affinity to HIV gp41 antigen were successfully obtained after two rounds of positive-screening and 4 rounds of counter-screening. Analysis of positive-screening and counter-screening using qPCR in the third round of counter-screening showed that the screened aptamer began to be enriched in positive-screening tube. Although the specific aptamers binding with the fixed target gradually increased in ssDNA library, aptamer enrichment did not reach saturation. After the fourth round of counter-selection, the specific aptamers binding with HIV gp41 antigen reached saturation in the positive-selection tube. Subsequent StEP and K_d value

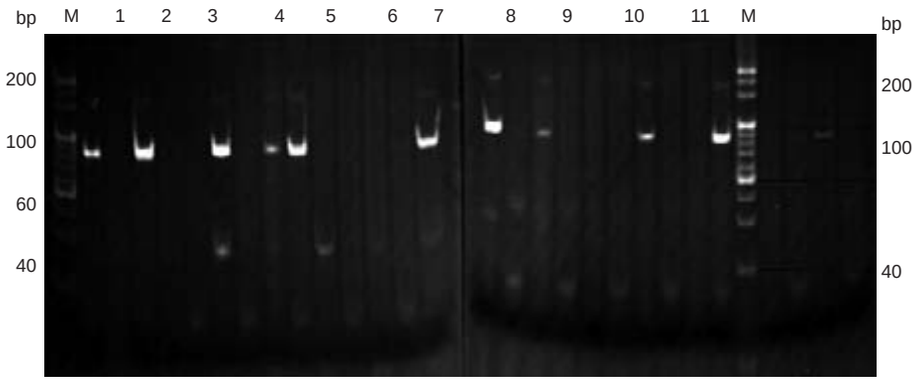

Fig.6 Electrophoresis of 9 positive monoclines identified using StEP technique. Lanes 6, 11: Blank control group. M: Marker.

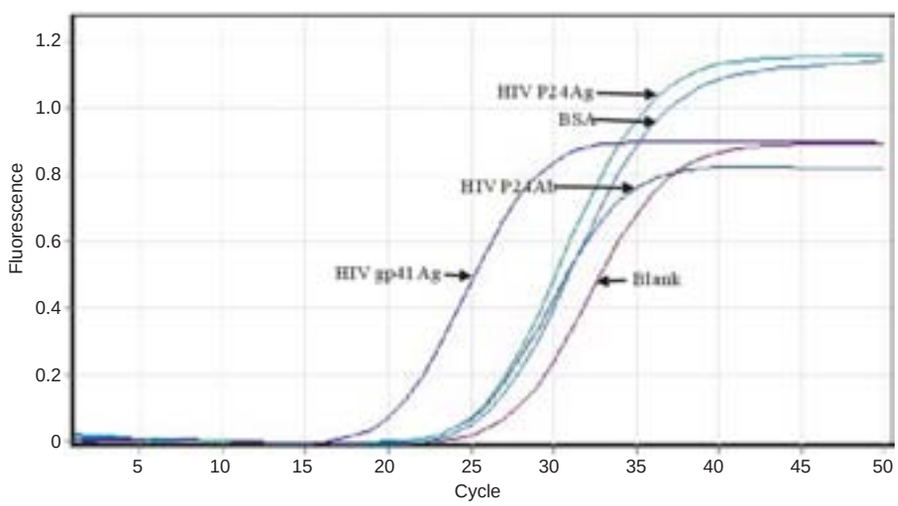

Fig.7 Identification of the specific aptamer of HIV gp41.

measurement indicated that the aptamers obtained by magnetic beads-based SELEX technique possessed a high specificity to HIV gp41 antigen. The utilization of qPCR, gel electrophoresis and other conventional techniques simplifies the procedures for screening the aptamers and shortens screening time. The aptamers obtained may facilitate future high-throughput screening and future development of rapid test kits of HIV with a low cost.

In this study, the aptamers with a highly specific affinity to HIV gp41 antigen were successfully obtained after 6 rounds of screening with magnetic beads. To reduce the non-specific adsorption of ssDNA as much as possible to obtain highly specific aptamers, the coupled targets were replaced in the counter-selection tube in the third round of screening. The specific aptamer obtained provides an efficient, rapid and accurate probe for early diagnosis of HIV by detecting the gp41 antigen.

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琼脂磁珠消减SELEX技术筛选HIV gp41抗原适配体

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摘要:目的 通过消减SELEX技术筛选得到高特异性、强亲和力的HIV gp41抗原适配体,为HIV的早期诊断提供了新的检测技术。**方法** 以琼脂磁珠为载体,HIV gp41抗原为靶标分子,利用消减SELEX技术和实时定量-PCR技术,筛选得到HIV gp41抗原适配体。**结果** 通过6轮筛选,筛选得到的次级ssDNA库通过PCR扩增得到dsDNA,dsDNA与pMDTM 18载体链接,进行克隆、测序,获得4条HIV gp41抗原适配体。获得的4条适配体亲和力 (Kd)均在纳摩尔水平,其中15号适配体的亲和力最强,特异性检测表明筛选得到的适配体几乎只与HIV gp41抗原结合,不与其他非特异性蛋白结合。**结论** 利用随机单链寡核苷酸文库成功获得与HIV gp41抗原特异性结合的适配体,所获得的适配体具有拮抗HIV gp41抗原的能力。

关键词:HIV gp41抗原;消减SELEX技术;DNA适配体识别

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