

In Vitro Selection of High-Affinity DNA Ligands for Human IgE Using Capillary Electrophoresis

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Aptamers with high affinity for IgE were selected using capillary electrophoresis to demonstrate the compatibility of this technique with SELEX. The high selectivity and efficiency of CE gave rise to a very high rate of enrichment, allowing high-affinity, high-selectivity aptamers to be obtained in only four rounds of selection. Decreasing the number of rounds shortens the selection procedure from the 4–6 weeks typical of SELEX to several days. The use of “bulk” dissociation constant measurements was introduced as a method for assessing the DNA pool after each round of selection. The average dissociation constant of the sequences in the DNA pool for IgE after four rounds of selection was 29 nM. The distribution of the dissociation constants for the sequences in the pool was very narrow with a standard deviation of only 6 nM. All of the sequences assessed exhibited high specificity for human IgE when compared with human IgG or mouse IgE.

SELEX, also referred to as in vitro selection or evolution, is a process for selecting high-affinity nucleic acid ligands from large DNA or RNA libraries.^{1–3} The SELEX process is highly parallel, allowing libraries with as many as 10^{15} constituents to be queried in a reasonable period of time. A number of reviews have been published that describe SELEX in detail.^{4–6} Briefly, a 10^{13} – 10^{15} member library of DNA or RNA containing 20–60 base random sequence regions is generated. Sequences that have affinity for the target are separated from nonbinding sequences using affinity chromatography, filter, or panning assays. Binding sequences are amplified using PCR to produce a new nucleic acid pool. This DNA or RNA pool can be further refined using additional rounds of selection. After sufficient selection, sequences are cloned and sequenced, yielding DNA or RNA molecules with high affinity for the target. These sequences, referred to as aptamers, typically have low-nanomolar dissociation constants for large targets such as proteins and micromolar dissociation constants for smaller molecule targets. Initially, SELEX received much attention as an alternative to current combinatorial methods for drug discovery. More recently, aptamers have found increasing use in chemical analysis. Aptamers have already been employed in a range of

analysis techniques including affinity capillary electrophoresis,^{7–10} capillary electrochromatography,^{11,12} HPLC,^{13,14} fluorescence,^{15–17} and AFM,¹⁸ suggesting that SELEX will have a significant impact on the analytical chemistry community.

The rate of enrichment between SELEX rounds is determined by the resolution of the separation of binding from nonbinding sequences. While affinity chromatography, filters, and panning separation schemes may provide high selectivity, the efficiency of these techniques is usually low, leading to poor separation and relatively low rates of enrichment. A typical SELEX process requires 8–12 rounds of selection to obtain high-affinity aptamers.¹⁹ This is a labor-intensive process often taking 4–6 weeks to complete. Other concerns regarding the selection process include the bias introduced by stationary-phase linkers in chromatographic selections, the poor kinetics of eluting very strong binders, and the potential for nonspecific binding with the support surfaces of the stationary phase, filter, or microtiter plate.

We have recently introduced capillary electrophoresis (CE) as an alternative selection mechanism for SELEX.²⁰ The new process, which we have termed CE-SELEX, selects binding sequences based on a mobility shift induced by complex formation with the target. In preliminary experiments, we demonstrated that high-affinity aptamers could be obtained in as little as two rounds of selection, shortening the selection procedure from several weeks to 2 days. The current paper describes the CE-SELEX process in more detail using IgE as a model selection target.

EXPERIMENTAL SECTION

Chemicals. All samples and buffers were prepared using deionized water obtained from a Milli-Q water purification system

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(Millipore Corp., Bedford, MA). The DNA library, PCR primers, and IgE aptamers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The DNA library consisted of two 20-base primer regions (necessary for PCR amplification) and a 40-base random region (5'-AGC AGC ACA GAG GTC AGA TG-(40 random bases)-CCT ATG CGT GCT ACC GTG AA-3') dissolved in the CE separation buffer (8.1 mM Na₂HPO₄ (Mallinckrodt, Paris, KY), 1.1 mM KH₂PO₄ (J. T. Baker, Phillipsburg, NJ), 1 mM MgCl₂ (Mallinckrodt), 2.7 mM KCl (Mallinckrodt), and 40 mM NaCl (Spectrum, Gardena, CA) at a pH of 8.0. Before selection, the 2.5 mM DNA library was incubated at 75 °C for 10 min and then slowly cooled to room temperature. Human myeloma IgE (monoclonal, κ , Athens Research and Technologies, Athens, GA) was diluted in CE separation buffer and added to the DNA library to give a final IgE concentration of 50 pM. The mixture was kept at room temperature for 30 min to allow complete binding to occur.

Capillary Electrophoresis. CE selections and ACE K_d measurements were performed on a commercial CE system (P/ACE MDQ, Beckman Coulter, Inc., Fullerton, CA). A 40.2-cm-long (30 cm to detector), 50- μ m-inner diameter, 360- μ m-outer diameter poly(vinyl alcohol)-coated capillary (Beckman eCAP N-CHO capillary, Beckman Coulter, Inc., Fullerton, CA) was used for the separations. Each day, capillaries were initially flushed with CE run buffer for 15 min. The separation capillary was rinsed with CE run buffer for 2 min before each separation. A potential of -20 kV (i.e., the inlet was the cathode) was applied during the separations. The current observed during separations was $\sim$ 70 μ A. The capillary cartridge was maintained at 25 °C. Samples were housed in a 4 °C chamber prior to injection. For CE-SELEX selections, samples were injected on to the capillary by applying 5 psi for 5 s. CE-SELEX selections were monitored using UV detection at 254 nm. After nonbinding sequences migrated off the capillary, CE fractions containing active DNA sequences were collected into a sample vial containing 48 μ L of deionized water by applying 10 psi pressure for 1 min. For K_d determinations 5-s, 1 psi injections were used and separations were monitored using laser induced fluorescence detection (λ_{ex} = 488 nm, λ_{em} = 520 nm).

PCR and ssDNA Formation. After collection, CE fractions containing binding sequences were amplified using PCR. PCR reagents were added to the CE fraction to give 1 mM concentration of each deoxyribonucleotide triphosphate, 1.5 μ M primer 1 (5'-AGC AGC ACA GAG GTC AGA TG-3'), 1.5 μ M primer 2 (5'-biotin/-TTC ACG GTA GCA CGC ATA GG-3'), 0.15 unit/ μ L Taq enzyme, and 7.5 mM MgCl₂. Denaturation was performed for 5 min at 94 °C. Note that denaturation disrupts DNA secondary structure, which induces strongly binding sequences to release the target molecule. A total of 18 cycles of denaturation (30 s, 94 °C), annealing (30 s, 53 °C), and extension (20 s, 72 °C) were performed followed by a final extension for 5 min at 72 °C. Control PCR amplifications were performed with all the PCR reagents listed above in the absence of any added DNA. The PCR reaction was verified by analyzing aliquots on a 2% agarose gel stained with ethidium bromide.

Amplified DNA was made single stranded using a streptavidin-agarose (Pierce Biotechnology, Rockford, IL) column. The streptavidin-agarose column was washed and equilibrated with binding buffer (10 mM Tris, 50 mM NaCl, and 1 mM EDTA at

pH 7.5) for 5 min. dsDNA from the PCR reaction was added to the equilibrated streptavidin-agarose column and held for 30 min with occasional shaking. The column was then washed 10 times with 1 mL of binding buffer. dsDNA was retained through the biotinylated complementary strand. The desired ssDNA sequences were eluted off the column using 200 μ L of 0.15 M NaOH at 37 °C and concentrated by ethanol precipitation. The biotinylated complementary sequences remained bound to the streptavidin-agarose column.

Clones of the ssDNA pool were prepared after four rounds of CE-SELEX selection. An aliquot of the ssDNA solution was PCR amplified. The PCR reagent mix and cycling conditions were similar to those described above, except that primer 2 was nonbiotinylated (5'-TTC ACG GTA GCA CGC ATA GG-3') and only eight PCR cycles were performed. Final extension was carried out for 10 min at 72 °C. The amplified DNA was cloned and sequenced by the BioTechnology Resource Center at the University of Minnesota (St. Paul, MN). Briefly, 50 μ L of the amplified DNA pool was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). The PCR product was ligated into the pGEM vector (Promega, Madison, WI). This ligation was transformed into DH5 *Escherichia coli* and colonies were raised. Plasmids from 32 colonies chosen at random were sequenced using the T7 promoter primer.

Dissociation Constant (K_d) Measurements. Dissociation constants were measured using affinity capillary electrophoresis (ACE).^{21,22} The separation buffer was identical to that used in the CE-SELEX selections. Samples containing 5 or 10 nM 6-carboxy-fluorescein (6-FAM)-labeled aptamers were incubated with varying concentrations of human IgE, human IgG, or mouse IgE and then injected onto the capillary.

The dissociation constant (K_d) was found by fitting the heights of the unbound aptamer peaks (I) to

$$\frac{I_0 - I}{I_0} = \frac{\text{constant}}{K_d + [\text{IgE}]} \quad (1)$$

where I_0 is the height of the unbound peak in the absence of IgE. Nonlinear least-squares regression analysis was performed to estimate K_d (Prism version 4.00 (Prism, v. 4.00, GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Figure 1 shows a schematic of the CE-SELEX process. The DNA library is incubated with the target in a sample vial. The sample is injected onto the capillary and separated using free-solution CE. As a first approximation, DNA migrates with the same mobility in free-solution CE, regardless of size or sequence. This is an advantage in CE-SELEX since all unbound DNA migrates as a single band. The formation of a complex with the target alters the mobility of the DNA, causing bound sequences to migrate at a different rate. It is a relatively simple matter of collecting the two bands into separate collection vials to separate binding from

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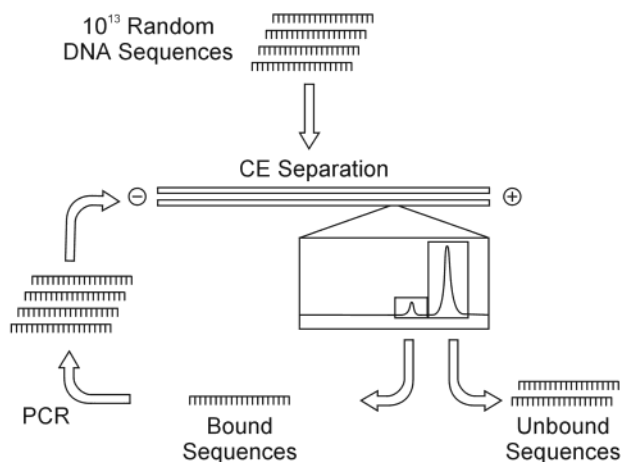


Figure 1. Schematic of the CE-SELEX process. A random DNA library is incubated with the target molecule; $\sim 10^{13}$ DNA sequences are injected onto the CE separation capillary. Bound DNA are collected and PCR amplified, preparing a new pool for further rounds of selection. After several rounds of selection, a high proportion of sequences in the DNA pool demonstrate high affinity for the target.

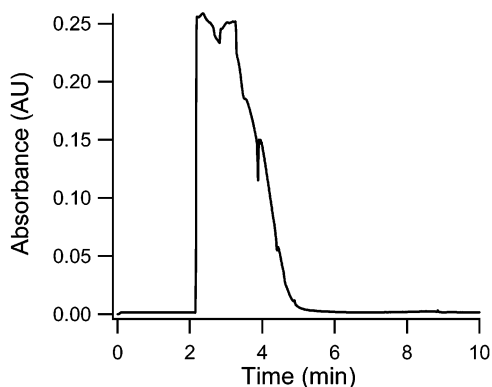


Figure 2. Electropherogram of the DNA library observed during the first round of CE-SELEX selection. The injection consisted of 2.5 mM DNA library and 50 pM human IgE. CE conditions: PBS buffer with 1 mM MgCl_2 , pH 8.0, 5-s/5 psi injection, 498 V/cm, 25 °C, UV detection at 254 nm. Only the unbound DNA are observed in the electropherogram. IgE–DNA complexes migrate at times > 10 min. Note that due to the low number of IgE molecules injected even if complete binding were to occur, the peak for the IgE–DNA complex would be below the limit of detection.

nonbinding sequences. The other steps of the process are similar to those of conventional SELEX. PCR is used to amplify the sequences that show affinity for the target. ssDNA is isolated and purified following PCR to generate a new nucleotide pool for subsequent rounds of CE selection.

There were some initial concerns regarding scaling SELEX down to dimensions typical for CE. Libraries in SELEX typically contain 10^{14} – 10^{15} independent nucleic acid sequences.^{4–6} This number is small compared to the number of possible sequences, minimizing the probability of replicate sequences being present. For example there are 10^{24} (i.e., 4^{40}) possible sequences in a library containing a 40-base random region. While it is not practical to create comprehensive libraries, large libraries do increase the probability that high-affinity ligands will be present. Even a relatively large CE injection volume of 50 nL only loads 7.6×10^{13} sequences when the concentration of DNA in the library is 2.5 mM. This high concentration of DNA gives rise to broad,

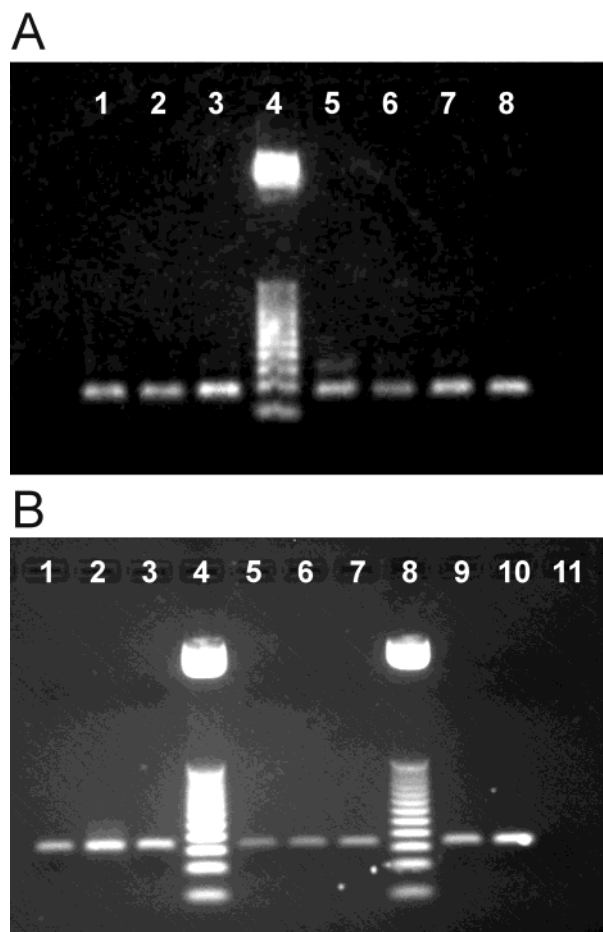


Figure 3. Gel electrophoresis analysis of PCR-amplified CE fractions collected during CE-SELEX selections of the DNA library. The library in (A) contained the primers first used by Wiegand et al. in the original IgE SELEX selection.²⁴ Primers with higher melting points were used in (B). In (A), PCR samples are in lanes 1–3 and 5–7. Lane 4 is a 25-bp DNA stepladder, and lane 8 is a PCR control. In (B), PCR samples are in lanes 1–3, 5–7, 9, and 10. Lanes 4 and 8 are 25-bp DNA stepladders, and lane 11 is a PCR control. Separation conditions: 2% agarose gel stained with ethidium bromide, TBE buffer, 40 min at 120 V. Bands migrated from top to bottom.

misshapen peaks when injected onto the CE (see Figure 2). These peak shapes are not surprising considering the high ionic strength of a 2.5 mM solution of DNA. Destacking undoubtedly occurs. Although the peak shape was poor, excellent separation was still achieved between the uncomplexed DNA sequences and the IgE complex. The pI of human IgE is ~ 9 ,²³ 1 pH unit higher than the CE separation buffer. Therefore, IgE is slightly positive at the separation pH, dramatically slowing the mobility of the negative DNA upon binding. In a previous study, German et al. demonstrated that the mobility of an IgE–aptamer complex was so slow that pressure needed to be applied to the capillary inlet to observe the complex.⁷ In the current study, separation voltage was applied for 10 min, allowing the unbound DNA to migrate into a waste vial. The outlet of the capillary was then placed into a collection vial containing 48 μL of deionized water, and pressure was applied to push the DNA–IgE complexes off the capillary. Peak shape of the DNA library is a potential limitation of the CE-SELEX

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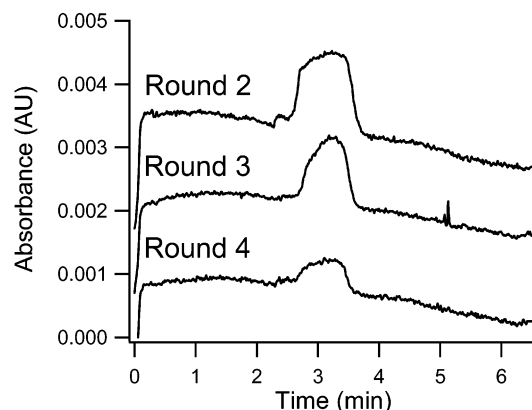


Figure 4. Electropherograms of the DNA pool observed during CE-SELEX selection rounds 2–4. DNA samples from the previous CE-SELEX round were incubated with 50 pM human IgE and separated using CE. CE conditions: PBS buffer with 1 mM MgCl_2 , pH 8.0, 5 s at 5 psi injections, 498 V/cm, 25 °C, UV detection at 254 nm. Electropherograms have been offset for clarity. Only the unbound DNA are observed in the electropherogram. IgE–DNA complexes migrate at times >10 min.

technique. We are currently assessing lower concentration libraries, which give much better peak shapes.

A second concern was scaling PCR down to account for the small amount of analyte typical in CE. In the current experiment, the concentration of target (IgE) in the sample vial was 50 pM. Assuming an injection volume of 50 nL, only 1.5×10^6 IgE molecules will be injected onto the separation capillary. Therefore, even if every IgE binds a DNA molecule, only 1.5×10^6 sequences will be collected for amplification. In earlier studies, we have used as few as 30 000 target molecules in a CE selection.²⁰ While using such small amounts of target increases the stringency of the selection, it puts high demands on the PCR amplification. Optimization of low-copy number PCR was an important step in making CE-SELEX feasible. Initial selection attempts were performed using primer regions identical to those described by Wiegand et al. (5'-CTA CCT ACG ATC TGA CTA GC (40-base random region) CCT TGA TGT ACT CTC ATT CG-3').²⁴ This was done to account for any possible contribution of the primer region to the binding motif, allowing a direct comparison between SELEX and CE-SELEX. Unfortunately, the primer regions used in the original IgE SELEX selection were not suitable for the low-copy number PCR required in CE-SELEX. Figure 3A shows a gel electrophoresis analysis of the PCR products obtained after a CE selection using a library containing the original primer sequences. The dsDNA sequences obtained were ~40 bases long, not the anticipated 80 bases. An identical band was observed in the control PCR where no collection step was performed. It appeared that primer dimers were being amplified, not the selected DNA sequences. To minimize the formation of primer dimers, we prepared a ssDNA library that contained primer sequences with higher melting temperatures (5'-AGC AGC ACA GAG GTC AGA TG (40-base random region) AAG TGC CAT GCG TAT CC-3'). Higher annealing temperatures can be used in the PCR reaction with high-melting temperature primers. Primer dimer formation is greatly reduced at the higher annealing temperatures, resulting

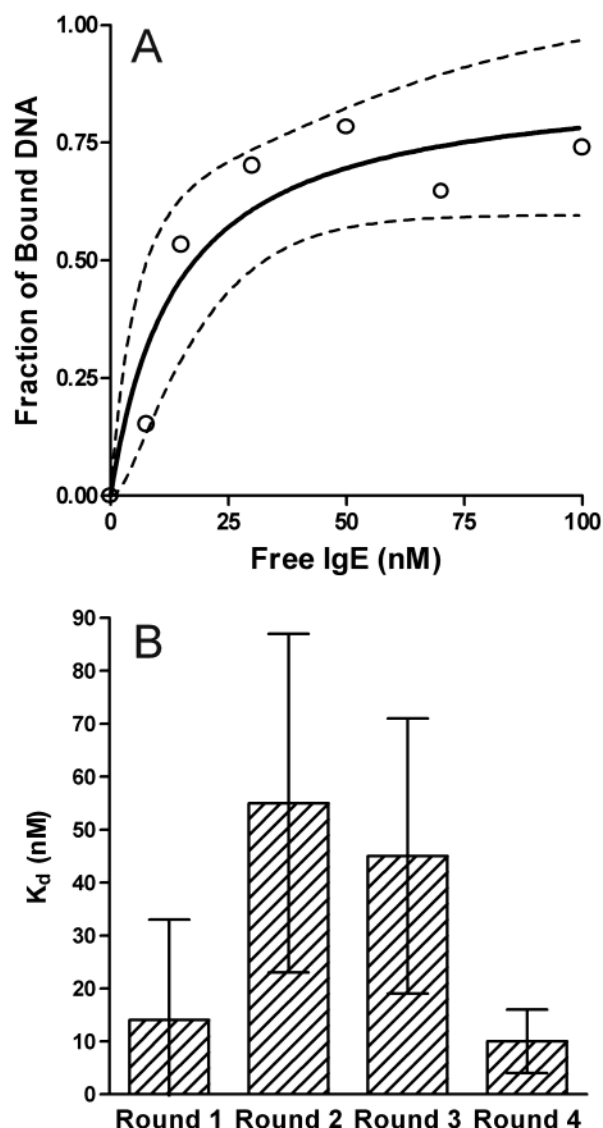


Figure 5. (A) Binding curve of the DNA pool after one round of selection. Each data point is the average of two replicate measurements. The solid line is the nonlinear least-squares fit of the data. The dashed lines are the 95% confidence limits of this fit. (B) Dissociation constants of the DNA pool after each round of selection. Error bars are the 95% confidence limits as determined from the nonlinear least-squares fit of the data.

in a more efficient amplification of the template DNA. The melting temperatures of the new 5' and 3' primers were 59.4 °C, which compared well with the melting temperatures of the original 5' and 3' primers, which were 51.8 and 50.7 °C, respectively. As shown in Figure 3B, the incorporation of the higher melting temperature primers into the library eliminated primer dimer formation. DNA sequences of the proper length were now observed.

Further rounds of selection were performed after the DNA pool was purified and made single stranded. Figure 4 shows electropherograms observed during subsequent rounds of selection. These electropherograms show that enough DNA is passed through the CE selection and collection process to sustain multiple CE-SELEX cycles. Again, peak shapes are poor due to overinjection of the sample. Relatively large injection volumes were used to maximize the amount of DNA used in each round of selection.

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Table 1. DNA Sequences Selected Using CE-SELEX To Have Affinity for Human IgE^a

clone	sequence (5' – 3')
4.4.1	AGCAGCACAGAGGTCAGATG TGCGGTTACATAAACTAACTGTGCCCCATTCTCATG CCTATGCGTGCTACCGTGAA
4.4.2	AGCAGCACAGAGGTCAGATG CGGCGCGGGGTCTTTTACAAATAGTTGTCTAGCCACG CCTATGCGTGCTACCGTGAA
4.4.3	AGCAGCACAGAGGTCAGATG ACTATTGCACCTGTTCCAGCTAACGTTGGTTTGGTAGGCC CCTATGCGTGCTACCGTGAA
4.4.4	AGCAGCACAGAGGTCAGATG ACATTTACAGCGTTTCATAGTAACCTGGCACTACAGTGCCG CCTATGCGTGCTACCGTGAA
4.4.5	AGCAGCACAGAGGTCAGATG CGATAGTGGTCGATTCTCCAACCTTTGTCTATGTGCACTTG CCTATGCGTGCTACCGTGAA
4.4.6	AGCAGCACAGAGGTCAGATG CTTCATTTCTCCGAGCTGTGACGACCGTATTTTCCTACG CCTATGCGTGCTACCGTGAA
4.4.7	AGCAGCACAGAGGTCAGATG TATCAGCATTGTAAATTTGGTACAATCTGTTTGGTTT CCTATGCGTGCTACCGTGAA
4.4.8	AGCAGCACAGAGGTCAGATG TATTGAGTCACTGTTTTAACTTTTCGTGGGGCCGGTT CCTATGCGTGCTACCGTGAA
4.4.9	AGCAGCACAGAGGTCAGATG TTAGCCCGCTCAGTCATCTGTAACCTCGTCACCTCATT TGCTATGCGTGCTACCGTGAA
4.4.10	AGCAGCACAGAGGTCAGATG CTATGAGCAGATCCGATAGAAAGTTAATGCCCTAGAACAT CCTATGCGTGCTACCGTGAA
4.4.11	AGCAGCACAGAGGTCAGATG AACCTCACAACTAGTTTTTGGTGTGCTTGGTCTCCGTT TGCTATGCGTGCTACCGTGAA
4.4.12	AGCAGCACAGAGGTCAGATG TGAAACATAGCATTTTACTTATGTCGCCCTTGCCGGTT CCTATGCGTGCTACCGTGAA
4.4.13	AGCAGCACAGAGGTCAGATG TTTCATCATTGTATCTGGGTTTCATGTGAGCACAAGGTGT CCTATGCGTGCTACCGTGAA
4.4.14	AGCAGCACAGAGGTCAGATG TAGGATTTTCTCAGACCGATACGCGTCGCTGTGCCTAGT CTATGCGTGCTACCGTGAA
4.4.15	AGCAGCACAGAGGTCAGATG CTCAAAGCGGGCGACTCTCAGGAACGCGGCTCAATT ACCCCTATGCGTGCTACCGTGAA
4.4.16	AGCAGCACAGAGGTCAGATG AAATTTGACCTTACGTTTCTCGATAGCCACAAG CTATGCGTGCTACCGTGAA
4.4.17	AGCAGCACAGAGGTCAGATG TGCTTATTTAGTCCCATGGTCAATTGCTGCGTATATAGT GCTATGCGTGCTACCGTGAA
4.4.18	AGCAGCACAGAGGTCAGATG TGCGATAAATAAATATTACACTGCGAATTTAGTAAAGGT GCTATGCGTGCTACCGTGAA
4.4.19	AGCAGCACAGAGGTCAGATG TAGGACCTCAATACACAGCTGTGTTTATGAATAAT TCCCTATGCGTGCTACCGTGAA
4.4.20	AGCAGCACAGAGGTCAGATG TGGTTCCAGCTGCAAGGTGTTGTCATTTGGCATATGTT ACCTATGCGTGCTACCGTGAA
4.4.21	AGCAGCACAGAGGTCAGATG ACGATCTCAGGTGATTAGCCGAGCTGTGTTTCAAAC TAACCTATGCGTGCTACCGTGAA
4.4.22	AGCAGCACAGAGGTCAGATG TGTCAGACGATCCGCTCGATATAGAGGCATTTCATTT CGGACCTATGCGTGCTACCGTGAA
4.4.23	AGCAGCACAGAGGTCAGATG TGTGTGAGGTTCAAAAACGTGCCACGTTAAAGACCGG TCTATGCGTGCTACCGTGAA
4.4.24	AGCAGCACAGAGGTCAGATG TACGGGAACCATCGTGAGGTGAACATACATACTG CCGCTATGCGTGCTACCGTGAA
4.4.25	AGCAGCACAGAGGTCAGATG TGGGGTTTTGGTAGCCTTTGCAATCCTTATGCGTCTGAGCC CTATGCGTGCTACCGTGAA
4.4.26	AGCAGCACAGAGGTCAGATG TCAAATATGTAATGTAGGTCCGTTTCGGGTGGGCCATGGG CCTATGCGTGCTACCGTGAA
4.4.27	AGCAGCACAGAGGTCAGATG AGATGGTTGACCGTTGATGTGATTAAGGAGACGTCGATT CTATGCGTGCTACCGTGAA

^a Primer regions are shown in boldface type.

This was made possible by the excellent resolution of the unbound DNA and the DNA–IgE complex. As with the initial round, voltage was applied for 10 min to elute the unbound DNA into a waste vial. Pressure was then applied to collect DNA–IgE complexes remaining on the column.

In previous work, we have demonstrated that nearly 100% of the DNA pool exhibits affinity for the target after only two rounds of selection.²⁰ Even though essentially all of the DNA pool already has affinity for IgE, it is possible that the strength of the binding interaction will continue to improve with further rounds of selection. In the current experiment, 1.5×10^6 IgE and $\sim 8 \times 10^9$ DNA molecules are injected onto the separation capillary for each round of selection. DNA molecules must compete for binding sites on the IgE since it is in such a large excess. This competition will select the best binders from the pool, further increasing the binding efficiency of the pool in subsequent rounds of selection. We have performed “bulk” binding measurements to assess the average K_d of the DNA pool as it goes through additional rounds of selection. After each round of selection, an aliquot of the DNA pool was fluorescently labeled by PCR amplifying a small volume in the presence of 6-FAM-labeled 5'-primer. Fluorescently labeling the pool allows lower concentrations to be used in the binding experiments, which in turn allows lower dissociation constants to be measured. Figure 5A shows the binding curve of the DNA pool after only one round of selection. The measured K_d is already 14 nM. Figure 5B shows the trend in “bulk” K_d measurements with further rounds of selection. Surprisingly, the dissociation constant increased after two rounds of selection. This may in part be due to the fact that only $\sim 60\%$ of the pool can bind the target after one round of selection, while almost 100% demonstrates affinity for IgE after two rounds of selection.²⁰ The bulk K_d is a weighted average of the dissociation constants for the sequences

Table 2. Dissociation Constants of Sequences Chosen at Random from Table 1 with Human IgE, Human IgG, and Mouse IgE^a

sequence	K_d		
	human IgE (nM)	human IgG (μ M)	mouse IgE (μ M)
4.4.2	30 $\pm$ 22	>20	>2
4.4.10	33 $\pm$ 36	>20	>2
4.4.12	23 $\pm$ 12	>20	>2
4.4.16	39 $\pm$ 18	>20	>2
4.4.20	25 $\pm$ 20	>20	>2
4.4.27	23 $\pm$ 13	>20	>2

^a Errors represent the 95% confidence interval as determined from nonlinear least-squares regression.

capable of binding the target. If a sequence has no affinity for the target, it has no effect on the curve. Therefore, if the sequences that had no affinity for IgE after one round were replaced by weakly binding sequences during the second round, this would diminish the observed bulk affinity. The bulk K_d measurements decreased with further rounds of selection as expected.

An aliquot of the DNA pool after the fourth round of selection was cloned and sequenced. Table 1 lists the sequences of the clones. Six clones were chosen for further analysis using a random number generator. This random sampling approach allows inferences to be made about the entire DNA pool, not just individual sequences. The six sequences were synthesized and their affinity for IgE was measured using ACE. Table 2 lists the dissociation constants measured for these six sequences. The dissociation constants for all of the sequences were tightly clustered in the low-nanomolar range. We had expected a much wider range of

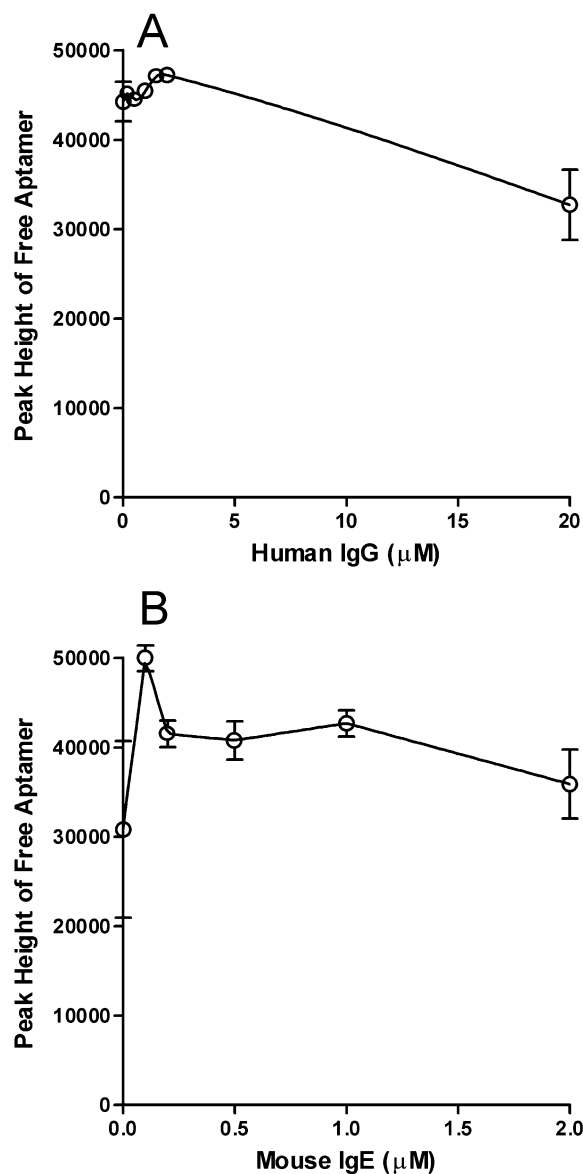


Figure 6. Binding curves demonstrating the selectivity of aptamers obtained using CE-SELEX for IgE over human IgG (A) and mouse IgE (B). Data for sequences 4.12 and 4.22 are shown in (A) and (B), respectively. Error bars represent the standard deviation.

values. The average dissociation constant in the DNA pool after four rounds of selection was 29 nM. The standard deviation of the distribution was only 6 nM, suggesting that most of the sequences in the pool exhibit remarkably similar affinity for IgE after four rounds of selection. It should be noted that we could not identify any significant sequence homology or motifs in the clones listed in Table 1, suggesting that there are many DNA sequences capable of binding IgE with high affinity. We should also note that the motifs identified by Wiegand et al. in the original IgE SELEX selection were also not observed.²⁴

The specificity of the sequences listed in Table 2 was assessed by measuring their affinity for human IgG and mouse IgE, two

proteins similar to human IgE. Panels A and B of Figure 6 show the binding curves obtained with increasing concentrations of human IgG and mouse IgE, respectively. No significant binding was observed up to the maximum concentrations measured, suggesting that the dissociation constants with human IgG and mouse IgE were even higher. Based on these estimates, the selectivity of the sequences listed in Table 2 for human IgE over human IgG and mouse IgE were >700 and >70 , respectively. It should be noted that nothing in the methods used here specifically selected sequences with selectivity for human IgE over human IgG or mouse IgE. It appears that selectivity is a fortuitous byproduct of the very high affinities observed here.

CONCLUSIONS

We have demonstrated that CE can be used as a selection mechanism in SELEX. Indeed, there are several advantages to doing so. Almost 100% of the DNA pool demonstrated affinity for IgE after only two rounds of selection. This is significant considering it often takes 8–12 rounds of selection in conventional SELEX to get a substantial fraction of the library to bind the target. There are several possible explanations for the improved rate of enrichment observed in CE-SELEX. Separation selectivity and efficiency is much higher in CE than in affinity chromatography or filter assays. To perform a selection, it is necessary to select several thousand (or hundred) binding sequences out of 10^{13} inactive sequences. The higher the resolution between binding and nonbinding sequences, the less contamination there will be during fraction collection and the higher the rate of enrichment will be. A second possibility is that there are less opportunities for nonspecific interactions to take place in CE. There are many sites on the stationary phases of affinity columns or filters at which nonspecific interactions could take place. Reducing these interactions again improves the purity of the binding DNA after fraction collection, increasing the rate of enrichment. In practical terms, the improved rate of enrichment observed in CE-SELEX significantly decreases the number of rounds of selection needed to obtain high-affinity aptamers, reducing the time required to complete the SELEX process from several weeks to as little as 2–4 days. We have also observed an abundance of sequences with high affinity for IgE. Whether this is due to the CE-SELEX process as a whole or the specific experiment described here remains to be seen. A potential limitation of the CE-SELEX technique is the minimum size of the target molecule. The target must be large enough to induce a mobility shift when it binds DNA. We are currently studying target molecules smaller than IgE to determine how significant this limitation will be.

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