Novel Combinatorial Selection of Phosphorothioate Oligonucleotide Aptamers†

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ABSTRACT: A novel combinatorial approach is described for construction and screening of enhanced nuclease-resistant phosphorothioate DNA "decoys" or "aptamers." Aptamers have been selected that bind tightly to the nuclear factor for human IL6 (NF-IL6), a basic leucine zipper transcription factor involved in the induction of acute-phase responsive and cytokine gene promotors in response to inflammation. Using a random combinatorial selection approach and dNTP(αS)'s in the PCR amplification, we can select specific thio-substituted agents which have the highest specificity in binding to target NF-IL6. Using a 22-nucleotide-long duplex random library, nanomolar binding, specific 22-mer thiophosphate backbone substitution sequences (at dA positions only) were selected. These show a different consensus sequence than normal phosphate backbone CCAAT/enhancer binding protein recognition sites for NF-IL6. Unlike the wild-type 10-mer sequences, which bind 1 protein dimer/duplex, these 22-mer thiophosphate aptamers bind with a stoichiometry of 2 dimers/duplex.

Oligonucleotide agents have been shown to have promising therapeutic potential. Some of these agents are believed to operate via mechanisms such as the sequence-specific antisense translation arrest of mRNA expression or through direct binding to protein targets where they function as "decoys" or "aptamers". Decoys have been used in blocking the action of the inducible NF-*k*B[1] transcription factor (*1*, *2*). While oligonucleotide agents show promising therapeutic activity, various pharmacological problems must first be overcome. Oligonucleotides themselves are susceptible to nuclease digestion and are thus not stable enough for intravenous or oral administration. Various backbone modifications such as the phosphorothioates and phosphorodithioates render the agents more nuclease-resistant, yet are taken up efficiently by cells (*3*, *4*).

Unfortunately, oligonucleotides possessing high thiophosphate backbone substitutions appear to be "stickier" toward proteins than normal phosphate esters, attributable to nonspecific interactions. Based upon NMR structural studies of dithiophosphate-substituted oligonucleotides, we have suggested that one explanation for the higher affinity of the thio-substituted DNAs is the poor cation coordination of the polyanionic backbone (*5*)—sulfur, being a soft anion, does not coordinate as well to hard cations such as Na^+, unlike the hard phosphate oxyanion. The thio-substituted phosphate esters then act as "bare" anions, and since energy is not required to strip the cations from the backbone, these agents can in principle bind even tighter to proteins. Even in specific protein−nucleic acid contacts, sulfurization of the internucleotide linkages can lead to their enhanced binding (*6*, *7*). This can be quite important since most of the direct contacts between DNA binding proteins and their binding sites are to the phosphate groups (*8*). We need, however, to optimize the total number of thioated phosphates to decrease nonspecific binding to nontarget proteins and enhance only the specific favorable interactions with the target protein. Thio substitution can also lead to structural perturbations in the structure of the duplex (*5*), although monothiophosphates substituted in the DNA strand of DNA/RNA hybrids do not dramatically alter duplex structures (*9*, *10*). How then do we determine what phosphates to substitute in a specific DNA sequence in order to develop selectively thio-substituted agents which retain the highest specificity in binding to target proteins?

A recent advance in combinatorial chemistry has been the ability to construct and screen large random-sequence nucleic acid libraries for affinity to proteins (*11*−*13*). The nucleic acid libraries are usually screened by incubating the target protein with the library and then employing a method of separating the nonbinding species from the bound. The bound fractions are then amplified using the polymerase chain reaction (PCR) and subsequently incubated again with the protein for a second round of the screening process. These iterations are repeated until one is satisfied that the library is enhanced for sequences which have high affinity for the target protein. However, agents selected from combinatorial

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[1] Abbreviations: bZIP, basic leucine zipper; BSA, bovine serum albumin; CD, circular dichroism; C/EBP*β*, CCAAT-enhancer binding protein *β*; DNase I, deoxyribonuclease I; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; K_{obs}, observed binding constant; NMR, nuclear magnetic resonance; NF-*k*B, nuclear factor-*k*B; NF-IL6, nuclear factor for human IL6; dNTP(αS), dNTP with monothiophosphorylation of the α-phosphate of tripolyphosphate; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Taq, Amplitaq polymerase; TCD, tryptic core domain of NF-IL6.

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libraries of RNA and DNA have in the past had normal phosphate ester backbones and thus will generally be unsuitable as drugs because of their nuclease susceptibility. The effect of substitution of nuclease-resistant thiophosphates cannot be predicted since the sulfur substitution can lead to significantly decreased (or increased) binding to a specific protein (*6*). In this paper, we report a novel combinatorial approach involving the construction and screening of a phosphorothioate DNA library. Our target is the nuclear factor for IL6 (NF-IL6), a basic leucine zipper transcription factor involved in the induction of acute-phase responsive and cytokine gene promotors in response to inflammation (*14*).

METHODS

Library. A random combinatorial library of normal phosphoryl backbone oligonucleotides was synthesized by automated DNA synthesizer (Midland Certified Reagents, Midland, TX) that was programmed to include all four monomer bases of the oligonucleotide during the coupling of residues in a randomized segment. This synthetic library has PCR primer segments at the 5′ and 3′ ends flanking the randomized region and thus can be replicated and amplified by Taq DNA polymerase (Amplitaq, Perkin-Elmer). A 66-mer has been used with a 22 base pair random central segment flanked by 21 and 23 base pair PCR primer regions: 5′CAGTGCTCTAGAGGATCCGTGAC N_{22} CGAAGCTTATCGATCCGAGCG3′. The resulting library thus exists as a population with potentially 4^{22} (10^{13}) different sequences.

The oligonucleotide library with phosphorothioate backbone substituted at dA positions was then synthesized by PCR amplification of the 66-mer template using commercially available Taq polymerase and using a mix of dATP(αS), dTTP, dGTP, and dCTP as substrates (Pharmacia, Inc.). The PCR conditions for amplification of the starting random library include 40 μM each of dATP(αS), dTTP, dGTP, and dCTP, 500 μM $MgCl_2$, 2.9 μM 66-mer random template, 5 units of Taq polymerase, and 400 nM each primer in a total volume of 100 μL. PCR was run for 25 cycles of 95 °C/1 min, 72 °C/1 min. This polymerase is known to PCR-amplify a phosphorothioate backbone template (*15*) so long as the dNTP(αS)'s are limited to no more than three different bases in the mixture (*16*). It also acts stereospecifically to incorporate the S_P-diastereomers of dNTP(αS)'s and is believed to yield the R_P stereoisomer as is found for other polymerases (*17*).

NF-IL6. When full-length NF-IL6 complexed with DNA is exposed to trypsin, a 9.5 kDa fragment was identified as the smallest fragment stably resistant to proteolysis. This basic leucine zipper (bZIP) domain peptide spans amino acids A^{266}−C^{345}, and is termed the tryptic core domain (TCD). High-level expression of recombinant NF-IL6 bZIP region in *E. coli* was achieved in the T7 promoter/polymerase system. The TCD expressed as a nonfusion protein constitutes 30% of the total soluble *E. coli* protein and was purified as previously described (*18*). The TCD bZIP domain binds DNA in a manner indistinguishable from full-length NF-IL6. Electrospray mass spectrometry indicates that the mass of TCD is 18 926 Da. These data indicated that TCD is a covalently linked dimer through its C-terminal disulfide bond (independently confirmed by analytical ultracentrifugation; unpublished experiments of Dr. J. C. Lee). Our selection experiments, however, are performed under conditions in which the disulfides are reduced and the TCD exists as a noncovalent dimer.

NF-IL6 Screening. This random library was screened to determine sequences, which have affinity to the bZIP domain of NF-IL6. PCR amplification of the single-stranded library provided chiral duplex phosphorothioate 66-mer at all dA positions. The filter-binding method was used for enrichment of binding sites (*19*). The PCR-amplified random library of the chiral duplex phosphorothioate 66-mer at dA positions (100 pmol) was incubated with 6.6 pmol of TCD in 50 μL of buffer containing 10 mM Tris, pH 7.5, 1 mM DTT, and 50−400 mM KCl and filtered through Millipore HAWP25 mm nitrocellulose filters [following a modification of the protocol from (*19*)]. Under these conditions, the DNA/protein complexes were retained on the filter. The filters had been previously presoaked in 10 mM Tris, pH 7.5, 1 mM DTT, and 50−400 mM KCl (1X binding buffer that contains no protein or DNA). The filter was then washed with 10 mL of 1× binding buffer to remove the majority of the DNA, which only weakly bound to the protein. A 1 mL solution of 8 M urea and 4 M NaCl was then added to elute the protein-bound DNA. A negative control without protein was performed simultaneously to monitor any nonspecific binding of the thiophosphate DNA library to the nitrocellulose filter. The DNA was ethanol-precipitated and once again PCR-amplified with the dATP(αS) nucleotide mix. Note, the PCR thermal profile was different than that used to make the starting library: 95 °C/1 min, 55 °C/1 min, 68 °C/1 min for 25 cycles. The PCR products were analyzed by 15% nondenaturing polyacrylamide gel electrophoresis.

At various stages of the selection process, the resulting libraries were cloned and plasmids from individual colonies sequenced. The normal phosphate ester 66-mer duplexes in the libraries were subcloned using the TA cloning kit (Invitrogen). As a control, four clones were also sequenced from the original combinatorial library and shown to have random sequence.

Fluorescence Polarization Assay. The affinities of the selected oligo's or libraries have been measured by fluorescence anisotropy (*20*). Fluorescence polarization titrations using increasing concentrations of the recombinant protein to bind a palindromic 5′-labeled fluorescein C/EBPβ 20-mer binding site with a normal phosphate ester backbone (dTGCAGATTGCGCAATCTGCA) gave an observed binding constant, K_{obs}, of 10 nM (data not shown).

Thiophosphate 66-mers were PCR-amplified, phenol-extracted, and ethanol-precipitated. DNA purity was >95% as assessed by PAGE gels. Varying concentrations of 66-mers, 5′-labeled fluorescein−20-mer palindromic binding site, and NF-IL6 TCD dimer were incubated in 10 mM Tris, pH 7.5, 50 mM KCl, 1.0 mM DTT buffer for 1 h prior to fluorescence polarization measurements. 66-mer concentrations were calculated at 20 OD(260)/mg. K_{obs} is the observed binding constant representing the 66-mer concentration providing a 50% decrease in the fluorescence polarization intensity change. Fluorescence polarization titrations were carried out on a Panvera Beacon polarimeter.

Nuclease Resistance. The sensitivity of the duplexes to DNase I degradation was monitored by native PAGE. Reaction mixtures contained either 40.5 or 33.8 μg/mL duplex (phosphoryl or thiophosphoryl, respectively) in 205 μL of 50 mM Tris, 10 mM $MgCl_2$, 50 μg/mL BSA, pH 7.5, buffer and 0.12 pg/mL DNase I (Sigma). Aliquots (20 μL) were removed at specific time points, and the hydrolysis was quenched by adding 4 μL of 0.5 M EDTA, 20 μL of 90% formamide, followed by boiling and storing at 0 °C. Gels were scanned, and the 66-mer lane was integrated using an Image1D gel scanner (Pharmacia).

RESULTS AND DISCUSSION

Aptamer Thiophosphate Library Selection. We have demonstrated that a thiophosphate backbone combinatorial library can be created by PCR methods with substitution of appropriate dNTP(αS) in the Taq polymerization step. This combinatorial thiophosphate duplex library was successfully screened for binding to the TCD of NF-IL6 by a filter binding method, modified to minimize nonspecific binding of the thiophosphate oligonucleotides to the nitrocellulose filter. The thiophosphate-substituted DNA can be eluted from the filter under high-salt, protein-denaturing conditions. Subsequent ethanol precipitation and another PCR thiophosphate amplification provide product pools for additional rounds of selection. To increase the stringency of binding of the remaining pool of DNA in the library and select tighter binding members of the library, the KCl concentration was increased in subsequent rounds from 50 to 400 mM. The stringency of selection was also manipulated by lowering the amount of protein as the iteration number increased.

The first selection experiment was carried through 7 iterations. Only 3 clones were selected and sequenced (Table 1) at this stage of the selection process. In all 3 early round clones (3:3), we found a general consensus sequence with a stretch of 8−11 A/C's: ACAACCC or ACACCACC. NF-IL6 is a CCAAT/enhancer binding protein (C/EBPβ) with specificity for two CCAAC/T boxes. Thus, in these early rounds of selection, the thiophosphate substitution at A has not dramatically altered the affinity for the "CAAC"-like box.

A second independent selection experiment was started from the original random combinatorial library and carried through 10 iterations, yielding the sequences shown in Table 1 (4 of 4 clones). As shown in Table 1, the two independent selection experiments identified a single unique sequence (compare clones 2 and 7). While the ACAACCC sequence once again appeared (clone 7), another unique new sequence (dGGGCCC GCTGT ACATG C ACACG, clones 4−6) was found for the entire 22 bp randomized segment. The table has been divided to emphasize homology among 5−6 bp consensus, putative recognition units: "GC-box"; "5′ACAGC·GCTGT"; "5′ACATG·CATGT"; "5′ACACG·CGTGT". Nascent elements of this new variation could also be observed even by round 7 of the first selection experiment (GC box, ACA, and ACACG units).

This 10th pool of the second selection experiment was carried through an additional 6 iterations, and in the 7 clones sequenced, 2 major sequence families were obtained (Table 1): (family A) dGGGCCC GCTGT ACATG C ACACG; and (family B) dCCG GTTGT TGTCC CACT CCACG. Within these 22-base sequences, only 1 or 2 base changes were found for each family (3 of the 7 were identical sequences; clones 8−10). Note that even by round 10, 3 members of the 22-base sequence are identical to the A family consensus sequence (clones 4−6). Family B retains the early round CAAC·GTTG consensus while family A has lost all "traditional" C/EBPβ CAAC/T box sequence homology. An additional group of 25 more clones were sequenced (data not shown), and the 22-mers were found to also fall within the two families (identical to the consensus sequence or differing by only 1 nt).

Table 1: Sequences of the Variable 22-mer Region in 66-mer Thiophosphate Aptamers, Selected after Indicated Rounds[a]

Consensus:	**"GC-box"**	**"ACAGC.GCTGT"**	**"ACATG.CATGT"**	**"ACACG.CGTGT"**
ROUND 7:				
1	5'd GCC	GTCC	ACATA CG	ACACCACC
2	5'dGGCC	GACCGC	ACA G C	ACAACCC
3	5'dGGC	GCGGAT	ACAACCC	ACACGC
2nd Independent Selection Experiment, ROUND 10:				
4	5'dGGGCCC	GCTGT	ACATG C	ACACG
5	5'dGGGCCC	GCTGT	ACATG C	ACACG
6	5'dGGGCCC	GCTGT	ACATG C	ACACG
7	5' dGGCC	GACCGC	ACA G C	ACAACCC
ROUND 16:				
Family A:				
8	**5'**GGGCCC	GCTGT	ACATG C	ACACG
9	**5'**GGGCCC	GCTGT	ACATG C	ACACG
10	**5'**GGGCCC	GCTGT	ACATG C	ACACG
11	**5'**GGGCCC	GCTGC	ACGTG C	ACACG
12	**5'**GGGCCC	GCTGT	ACACG C	ACACG
Family B:				
13	5' CCC	GTTGT	TGTCC CACT	CCACG
14	5' CCC	GTTGT	TGTCC CGCT	CCACG

[a] Sequences are aligned to highlight the consensus elements (underlined). All sequences are written such that the first six flanking 5′ and 3′ primer sequences are all 5′GCTTCG and 5′CTCACC, respectively.

These results differ from normal phosphate ester backbone in vitro selection experiments with NF-IL6, where a traditional CAAC box was identified using the same TCD of NF-IL6 and a 66-mer library under identical selection conditions as described in this paper (A. Brasier and S. Widen, unpublished). Osada et al. (*21*) using full-length C/EBPβ and a 16-nucleotide randomized library were able to determine a 10 bp consensus sequence showing the expected two-half-site GTTGC·GCAAC in a palindromic sequence:

Consensus phosphate ester (21)	GTTGC GCAAC
Consensus thiophosphate ester (Family A)	GCTGT ACATG
Consensus thiophosphate ester (Family B)	GTTGT CCCAC
	or GTTGT TGTCC

Alignment of a consensus sequence is more difficult for family B members. Clearly, thiophosphate substitution on dA only has altered the sequence selected, eliminating the

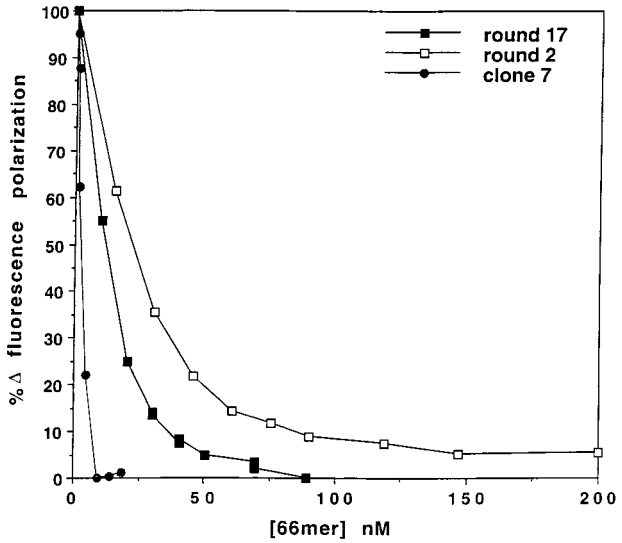

FIGURE 1: Competitive fluorescence polarization titration of 5′-labeled fluorescein−20-mer duplex/NF-IL6 TCD dimer complex. Percentage fluorescence polarization change plotted against concentration of added 66-mer thiophosphate duplexes. Clone 7 thiophosphate duplex, 1 nM 5′-labeled fluorescein−20-mer duplex, and 18 nM TCD dimer (●). Stoichiometric titration of second round selection, thiophosphate 66-mer library, 25 nM 5′-labeled fluorescein-20-mer, and 90 nM TCD dimer (□). Seventeenth round selection, thiophosphate 66-mer library, 25 nM 5′-labeled fluorescein−20-mer, and 90 nM TCD dimer (■).

sequential AA consensus sequence found in all other phosphate selection experiments. In both normal phosphate and thiophosphate duplex 10-mers, 4−5 dA's can be found, indicating that thiophosphate substitution for the dA residues has not had a deleterious effect on binding, other than eliminating sequences containing 2 adjacent thiophosphates.

Binding Affinity for NF-IL6. The monothiophosphate libraries and individual 66-mer sequences were used as competitors to dissociate a fluoroscein-labeled, normal backbone duplex C/EBPβ 20-mer bound to the bZIP protein. As shown in Figure 1, an individual monothiophosphate 66-mer cloned from the 10th selection round (clone 7) gave a K_{obs} of <2 nM. Following a similar competitive titration, monothiophosphate (at dA) clones 8 and 13 (consensus family A and B, respectively) also gave a K_{obs} of <2 nM. As a negative control, at a concentration of up to 4 μM, thiophosphate clone 8 (family A) was shown not to bind to another transcription factor, NF-κB (p65 dimer) (S. Fennewald, J. Aronson, and N. Herzog, unpublished).

Stoichiometric titration of 66-mers with TCD established that later rounds (Figure 1) bound the protein dimer with an approximately 2:1 (protein dimer:DNA duplex) stoichiometry. However, initial rounds bound to TCD with a 1:1 stoichiometry (Figure 1). This change in stoichiometry could explain the remarkable selection of a full 22-base sequence when the NF-IL6 consensus site is believed to be only 10 bases in length.

The crystal structures of leucine zippers such as GCN4 and AP-1 in the DNA cocrystal are coiled-coils (*22*) with the basic region coiling into a helix to bind in the major groove in each of the half-sites. In a bZIP protein such as NF-IL6, it would be expected that each basic region of the protein binds as an α-helix to the two CAAC/T half-sites in the generally palindromic recognition sequences. NMR and

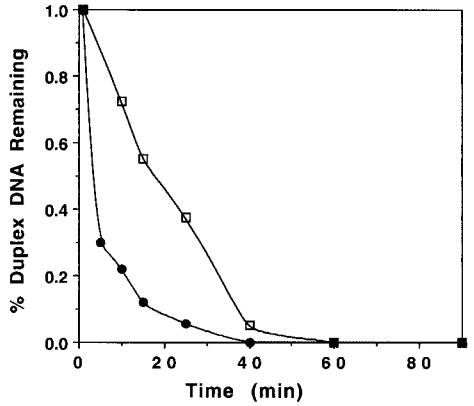

FIGURE 2: Relative sensitivity of family A 66-mers to degradation by DNase I. Unmodified, phosphoryl duplex (●) and monothiophosphorylated at nonprimer dA sites only (□).

CD studies of NF-IL6 TCD indicate that the basic region has a surprising degree of α-helical character, although CD studies indicate that there is a further increase in the α-helical content of TCD upon DNA binding (D. Volk and J. Wu, unpublished).

To explain the 2:1 stoichiometry for binding family A consensus 22-mer sequence, two TCD dimers must be capable of orienting on the duplex. Note that the family A 22-mer contains the consensus sequence:

5'dG G G C C C GCTGT **ACATG** C **ACACG**

C C C G G G **CGACA** TGTAC G TGTGCd5'

There are three very similar sequences shown above in boldface: 5′ACAGC, 5′ACATG, 5′ACACG [recall that these sequences are being selected even by rounds 7−10, and remarkably in the same orientation and order shown above (Table 1)]. This suggests that one TCD dimer can bind with each basic recognition helix interacting with each of the 5 nt sequences (containing ACA·TGT triplets) shown in boldface. The second TCD dimer could bind with one of the two basic recognition helices recognizing the third 5 nt sequence in boldface.

The six N-terminal residues of TCD are required for binding to the NF-IL6 consensus sequence (*18*). Although not part of the basic domain of the protein, our NMR structural studies show that there is considerable helical content in this region (data not shown). It is attractive to speculate that upon NF-IL6 binding to the full 22-mer, the N-terminal helical segment of one bZIP dimer interacts with an adjacent helical segment of another bZIP dimer, similar to the helix−helix stabilization of dimerization of the *lac* repressor headpiece to full *lac* operator DNA (*23*). The origin of the preference for the "GC-box" sequence at the 5′ end is unclear, and solution will require further combinatorial selection experiments with varying lengths of random libraries. Part of this specificity may also be related to the 5′ and 3′ sequences that flank the randomized 22-mer segment. It is interesting that the native NF-IL6 promotor site is positively regulated at low NF-IL6 concentrations and negatively regulated at high concentrations (*24*).

Nuclease Resistance. As shown in Figure 2 thiophosphorylation of the family A 66-mer at only the dA sites (except for the primers) results in a duplex that is more resistant to DNase I degradation than the unmodified 66-mer. These

results are consistent with previous studies showing enhanced nuclease resistance to both single-stranded and double-stranded DNA containing monothiophosphates (*16*, *25*−*27*). This is true for both endonucleases such as DNase I or exonucleases such as Bal 31, and, as expected, the greater the number of thiophosphates within the duplex, the greater the nuclease resistance (*16*).

CONCLUSIONS

These results demonstrate that oligonucleotide combinatorial methods can now be extended to selection not only of base sequence but also of phosphate (or monothiophosphate) backbones as well. The best monothiophosphate aptamers bind at least 5-fold tighter to NF-IL6 TCD as the normal backbone sequence. The sequences selected, while related to the normal backbone CAAC/T half-sites (for family B), show distinct differences that are likely attributable to alterations in the nature of the protein−phosphate backbone interactions in the complex. Because Taq polymerase can use up to three different dNTP(αS)'s in the polymerization reaction, further backbone substitutions are possible. We can readily incorporate both triphosphate and triphosphate-(αS) nucleotides in the PCR mix so that a library of both phosphate and monothiophosphate backbones can be randomized at the same base position, greatly increasing the diversity of the library. Since sequencing based upon thiophosphate substitution is possible (*15*), either backbone can be combinatorially selected and identified at any position. Recent advances in enzymatic and solid-phase synthesis of stereoregular monothiophosphate oligonucleotides (either *R* or *S* configuration at phosphorus for chemical synthesis) now permit large-scale synthesis of defined stereochemistry and sequence thiophosphate aptamers (*28*, *29*). Random combinatorial libraries and selection for aptamers with a much greater diversity of structures (7^N vs 4^N) are thus possible. Since thiophosphate substituted oligonucleotides show reduced nuclease activity and enhanced interaction with proteins in general, not just DNA binding proteins, this "thiophosphate-selection" experiment can offer wide application.

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