A Fluorescent Intercalator Displacement Assay for Establis\textbf{hing} DNA Binding Selectivity and Affinity

WINSTON C. TSE AND DALE L. BOGER*

Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

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ABSTRACT

A summary of the qualitative and quantitative elements of a fluorescent intercalator displacement (FID) assay useful for establishing the DNA binding selectivity, affinity, stoichiometry, and binding site size and distinguishing modes of DNA binding is provided.

Introduction

The regulation of gene expression is based on the sequence-selective recognition of nucleic acids by repressor, activator, and enhancer proteins. A full understanding of the proteins involved, the delineation of the sequences to which they bind, and the discovery of the genes that they regulate holds significant promise in therapeutic medicine.\textsuperscript{11-16} Thus, extensive efforts continue to be directed at understanding the transcriptional process and are being increasingly directed at the discovery of small molecules that selectively bind DNA and activate (block a repressor) or inhibit (block an activator) gene expression.\textsuperscript{7-11}

Of the techniques commonly used to establish the DNA binding properties of small molecules and proteins, most are technically challenging, require the knowledge of specialized biochemical procedures, and are time and labor intensive. The most widely used methods are footprinting\textsuperscript{12} and affinity cleavage.\textsuperscript{13} Because of the power of the technique, a number of such methods have been introduced including DNase I,\textsuperscript{14} exonuclease III,\textsuperscript{15} MPE linking\textsuperscript{21,22} (e.g., mitomycin) and FC-1065, duocarmycins), intrinsic DNA cleavage (e.g., bleomycin, endiynes), alkyl-ation/thermal cleavage\textsuperscript{20} for the iterative deconvolution of mixture libraries have been disclosed, expanding their applications. Inherent in these methods is the characterization of the highest affinity sites within a size-limited segment of DNA. Similarly, the DNA binding properties of proteins\textsuperscript{27} are typically assessed by selection screening,\textsuperscript{28-30} footprinting,\textsuperscript{14} or EMSA.\textsuperscript{31} The former provides exhaustive sequence coverage for deducing the preferred site(s), but it selects only the highest affinity sites and does not provide quantitative binding information. Footprinting and EMSA\textsuperscript{31} have been used to define, or at least refine, a protein’s binding selectivity, but their most frequent uses have been to provide qualitative distinctions and quantitative comparisons among candidate binding sites or those constructed to assess single base pair (bp) substitutions.

Herein, we review a complementary technique, a fluorescent intercalator displacement (FID) assay,\textsuperscript{32,33} for establishing DNA binding affinity, sequence selectivity, and binding stoichiometry. The assay is nondestructive, technically nondemanding, and amenable to high-throughput screening. The former feature would permit DNA immobilization onto reusable supports for repetitive use and expansion of the sequence space beyond that presently exemplified (all 5-bp sites). For a single compound, the technique permits establishment of a rank order binding profile for all possible 5-bp sites, comprehensively defining the sequence selectivity in a single experiment. For a defined sequence, it permits the high-throughput identification of binding agents from a library of compounds or quantitative titrations for establishment of binding constants. The assay is not limited to small molecule assessments and has been used with a variety of ligands, including proteins and triplex-forming oligonucleotides.

The FID Assay

The assay utilizes the displacement of ethidium bromide (or thiazole orange)\textsuperscript{34,35} from hairpin deoxyoligonucleotides (Figure 1). Hairpin DNAs are treated with the intercalator, yielding a fluorescence increase upon binding. Addition of a DNA binding compound results in a decrease in fluorescence due to displacement of the bound intercalator, where the percent fluorescence decrease is directly related to the extent of binding. For a panel of DNA sequences displayed in a 96-well format, the resulting profile of percent fluorescence decrease provides relative binding affinities and comprehensively defines a compound’s sequence selectivity. With individual sequences, quantitative titration of a compound against a hairpin prebound with ethidium provides reliable binding constants and the stoichiometry of binding. These two systems may be used in a complementary fashion, one intended for a high-throughput screen.

Dale L. Boger is the Richard and Alice Cramer Professor of Chemistry at The Scripps Research Institute with Professor Boger. He joined Gilead Sciences in 2003.

WINSTON C. Tse received a B.A. from Northwestern University, where he worked in the laboratory of Professor Joseph B. Lambert, and his Ph.D. from The Scripps Research Institute with Professor Boger. He joined Gilead Sciences in 2003.

* Corresponding author. Tel.: (858) 784-7522. Fax: (858) 784-7550. E-mail: boger@scripps.edu.
Fluorescent Intercalator Displacement Assay for DNA Binding

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FIGURE 1. The FID assay.

96-well plate with each well containing one individual sequence. The plate may contain library of all possible sequences to screen a single agent. The plate may contain one (or a few) select sequence(s) to screen a library of compounds.

Addition of ethidium bromide

Nonspecific intercalation results in fluorescence

Addition of a DNA binding compound

DNA affinity is measured as a decrease in relative fluorescence indicating binding with displacement of ethidium bromide.

Establish rank order binding. Define DNA sequence selectivity of a given compound or select compounds with affinity for a given DNA sequence.

FIGURE 2. Structures of hairpin deoxyoligonucleotides, ethidium bromide, and thiazole orange.

96-well plate with each well containing one individual sequence. The plate may contain library of all possible sequences to screen a single agent. The plate may contain one (or a few) select sequence(s) to screen a library of compounds.

5'-CGXXXXX A A
3'-GCXXXXX A A
X = A, T, G, C

Variable region: easily extendable from 5 base pairs (shown) to longer sequences

named 5'-ATGCA

5'-CG [ATGCA] C A A
3'-GC TACGT G A A

equiv 5'-CG [TGCA] C A A
3'-GC AGGTA G A A

* Each hairpin DNA contains both complementary 5' - 3' sequences
* Hairpins, and therefore sequences, are equivalent if the position of the variable region is not considered

with less sequence variation and greater consistency in the absolute fluorescence readings. However, the binding constants established with thiazole orange are slightly lower than those established with ethidium.32,39 This reflects the higher association constant and greater competitive binding of thiazole orange, reducing the measured apparent binding constant.

Hairpin deoxyoligonucleotides40 (Figure 2) proved especially useful in the FID assay. Embedded in the hairpin are two complementary 5'-to-3' sequences, connected by a loop, avoiding the requirement for two separate strands and the associated additional quantitation and mixing. The number of hairpins required to create a library of sequences is half the number of sequences. For example, 512 hairpins are required for a library of all 1024 possible 5-bp sequences (Figure 2). Moreover, the hairpins were established to provide stable duplexes at working temperatures (25 °C), independent of the sequence.32,33 The variable most critical to the success of the assay, and most likely to be responsible for avoidable errors, is the quality of the hairpins. In addition to the concern about their constitution and purity, their concentration is critical and may be determined by measuring the UV absorption (260 nm) of the denatured, single-stranded DNA at 80–95 °C.

Since the hairpins exist in a construct representing a combination of double- (stem) and single-strand (loop) DNA at 25 °C, calculations based on the UV absorption at 25 °C, utilizing the standard coefficients useful for single-stranded deoxyoligonucleotides, underestimate the concentration by as much as 25%.41

Binding Constants and Stoichiometry

Quantitative displacement of ethidium from a hairpin deoxyoligonucleotide provides a well-defined titration curve that is useful for establishing binding constants and
the stoichiometry of binding. A plot of the change in fluorescence versus equivalents of compound provides a titration curve from which the stoichiometry of binding may be derived as the intersection of the pre- and postsaturation portions of the curve (Figure 3). This method, analogous to that introduced by Bruice with Hoechst 33258,42,43 is easily extended to analyzing higher order 2:1 and 3:1 complexes. For 1:1 binding, binding constants are established by Scatchard analysis44 of the equilibrium portion of the titration curve, generating a plot of $\Delta F/\left[\text{free agent}\right]$ versus $\Delta F$, yielding a linear section where the slope provides $K_a$. Binding constants produced by this indirect technique involving the displacement of ethidium yielded results comparable to those obtained directly by monitoring the fluorescent increase of selected fluorescent DNA binding compounds (e.g., DAPI, Table 1).

DNA Binding Sequence Selectivity

Minor Groove Binding Compounds. In developmental stages of the assay, distamycin, netropsin, DAPI, Hoechst 33258, and berenil were examined (Figure 4).32 DAPI, Hoechst 33258, and berenil are fluorescent dyes, enabling a direct assessment of binding. Notably, the fluorescence enhancement characteristic of their binding did not interfere with the measurement of the fluorescence decrease derived from ethidium displacement (e.g., for EB, ex. 545 nm, em. 595 nm; for DAPI, ex. 372 nm, em. 454 nm).

Each compound was screened against a library of 512 DNA hairpins (1.5 $\mu$M) containing all possible 5-bp sites in a 96-well format, enlisting three compound concentrations (1.0, 1.5, and 2.0 $\mu$M) in duplicate and a Gemini SpectraMax plate reader.32 On this scale and at these concentrations, the cost of the purchased hairpins is approximately $100/assay. This provided a rank order binding profile for all possible 5-bp sites that is represented as a merged bar graph in Figure 5 for distamycin. In addition to rapidly providing a comprehensive definition of each compound’s sequence selectivity, the comparisons provided insights not previously easily recognized. While all compounds displayed the expected A/T binding selectivity, netropsin was the most A/T selective and, by some accounts, distamycin was the least. All exhibit tight binding to 5-bp > 4-bp > 3-bp A/T sites (Figure 6). This preference is related in part to the conformational characteristics of DNA, where longer A/T sites possess a narrower, deeper minor groove known to contribute to the selective binding. Interestingly, distamycin displayed an affinity for GC bp interrupted 5-bp A/T sites (2 x 2-bp) that exceeded even that of 3-bp A/T.

### Table 1. DAPI Binding Constants

<table>
<thead>
<tr>
<th>DNA sequence</th>
<th>$K \times 10^6$ M$^{-1}$</th>
<th>$K \times 10^6$ M$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-AATTTC</td>
<td>110</td>
<td>120</td>
</tr>
<tr>
<td>5'-AATTT</td>
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<td>52</td>
<td>77</td>
</tr>
<tr>
<td>5'-AAAAA</td>
<td>50</td>
<td>65</td>
</tr>
</tbody>
</table>

$^a$ Scatchard analysis of ethidium displacement titration. $^b$ Direct titration using fluorescence enhancement of DAPI.
sites and was only slightly weaker that that of 4-bp A/T sites. Netropsin exhibited a weaker preference for such 2 × 2-bp A/T sites, reduced from its affinity to 4-bp A/T sites. In constrast, DAPI, Hoechst 33258, and berenil exhibited a clear preference for a 3-bp A/T site over a 2-bp A/T site. Combined, this was suggested to reflect the larger 5-bp binding site requirement for distamycin and its unique compensating ability to bind selected GC bp interrupted 5-bp A/T sites. For each compound, binding constants were determined by ethidium displacement titrations with selected sequences. These were found to be comparable to those established by footprinting or calorimetry, and to be nearly identical (within 2-fold) to those obtained by directly monitoring the fluorescence increase of DAPI and Hoechst 33258 upon DNA binding (see Table 1).32

A similar screen of netropsin against a library of all possible 4-bp sites was disclosed, and binding constants for all 10 possible 4-bp A/T sites were determined by titrations (Table 2).30 With binding constants for all 10 possible A/T sites, an explicit rank order binding was established that was more comprehensive than that provided in all prior combined footprinting studies. Highlighted in these studies was the fact that the assay allows the rapid identification of the preferred ensemble of sequences for a compound, but that substantive conclusions drawn about adjacent sequences in the 96-well screen should be reserved for quantitative titrations.

Hairpin Polyamides. An analysis of four pyrrole (Py)/imidazole (Im) hairpin polyamides (Figure 7) was conducted that not only served to establish the utility of the assay for examining minor groove binding polyamides, but also underscored its comprehensive capabilities. The polyamides were chosen to examine the subtle effects of N- and C-terminal functionalization on the DNA binding selectivity, representing a challenging test of the assay
Table 3. Sequence Selectivity of ImPyPy-γ-PyPyPy-β-Dp

<table>
<thead>
<tr>
<th>Sequencea</th>
<th>1.5 μM agent</th>
<th>2.0 μM agent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg Score</td>
<td>Avg Score</td>
</tr>
<tr>
<td>5′-WGWWWWW</td>
<td>55 0.47</td>
<td>54 0.47</td>
</tr>
<tr>
<td>5′-WGGWWW</td>
<td>82 0.44</td>
<td>36 0.55</td>
</tr>
<tr>
<td>5′-WCWWWW</td>
<td>97 0.32</td>
<td>105 0.28</td>
</tr>
<tr>
<td>5′-WWWWWW</td>
<td>131 0.27</td>
<td>111 0.27</td>
</tr>
</tbody>
</table>

A. FID Assay Analysis

B. Binding Constants by FID Titration

<table>
<thead>
<tr>
<th>Binding Modeb</th>
<th>Sequence</th>
<th>Kd (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′-TCATTAAAC</td>
<td>1. 5′-TAACAC</td>
<td>2.39 × 10²</td>
</tr>
<tr>
<td>5′-TAACACC</td>
<td>3. 5′-TAACAC</td>
<td>1.33 × 10⁷</td>
</tr>
<tr>
<td>5′-GAACCT</td>
<td>4. 5′-GAACAC</td>
<td>3.31 × 10⁷</td>
</tr>
<tr>
<td>5′-GAAACC</td>
<td>5. 5′-GAAACC</td>
<td>2.84 × 10⁷</td>
</tr>
<tr>
<td>5′-ATTGTAA</td>
<td>6. 5′-ATTGTA</td>
<td>9.87 × 10⁷</td>
</tr>
<tr>
<td>5′-GTGTGTA</td>
<td>7. 5′-GTGTGA</td>
<td>9.89 × 10⁷</td>
</tr>
<tr>
<td>5′-GTGTGTC</td>
<td>8. 5′-GTGTGA</td>
<td>2.57 × 10⁷</td>
</tr>
<tr>
<td>5′-GTGTGTC</td>
<td>9. 5′-GTGTGC</td>
<td>3.30 × 10⁷</td>
</tr>
</tbody>
</table>

a W = A or T, b O, Py; c Im. Lit. 48 K = 2.9 × 10⁸ M⁻¹, footprinting.

capabilities. The complete binding profiles of the four molecules to all possible 5-bp sites were established using the FID assay.46

The comparison of 1 and 2 revealed the detrimental effect of the N-acetyl substitution with 2,46,47 and the hairpin polyamide ImPyPy-γ-PyPyPy-β-Dp (3), like 1, displayed a straightforward correlation with its expected selectivity for 5′-WGWWWWW. For ImPyPy-γ-PyPyPy-β-Dp (4), the ability to screen complete sequence space resulted in the discovery of an unexpected 5′-WWCWW sequence specificity (Table 3A). Although detailed studies have probed the ligand-DNA interactions of this molecule and its sequence selectivity was established (5′-WGWWWWW),48 its explicit binding to 5′-WWCWW had not been described.

Use of the FID assay to comprehensively screen DNA provided a complete picture of its selectivity. Strikingly, the quantity and quality of the data generated by the assay also provided the basis for a binding model. This model established that 4 has an optimal binding site of 6 bp (not the expected 5 bp) of the form 5′-WWGWWW, and that the β/Dp tail combination requires two (not one) degenerate A/T base pairs. FID titrations confirmed the model by providing quantitative binding constants (Table 3B).

Also disclosed in this study was the analysis of sequence sets and two scoring methods for their comparison (Table 3A). The first simply calculates the average rank order position of the constituent members of a sequence set, whereas the second calculates an average %F decrease of the sequence set relative to the highest affinity sequence. This latter, less obvious scoring procedure may better reflect the relative importance of binding to a sequence set.

Selection of Binding Agents for a Defined Sequence(s) from Compound Libraries

Most of the current screening technologies are sufficiently technically demanding that they are most often used to investigate a few individual compounds. A true attribute of the FID assay is its capabilities for screening libraries of compounds (mixtures or individual).33,49

The rapid screening of individual sequences was illustrated with two hairpin deoxyoligonucleotides containing two sequences of the androgen response element (ARE), the 14-bp ARE-consensus, and PSA-ARE-3.33,48 The emergence of hormone-independent, constitutively active androgen receptor dimer is responsible for prostate cancer relapse resistant to chemotherapeutic treatment. At this stage, competitive inhibition of the androgen receptor dimer DNA binding has therapeutic potential and could arise from small molecules with selective affinity toward the ARE-consensus and PSA-ARE-3. Libraries of distamycin-like compounds were prepared using 11 heteroaromatic subunits in addition to the N-methylpyrrole native to distamycin (Figure 8). Libraries were produced such that the first two subunits (B and C) were fixed, producing 132 mixtures of 10 compounds with variations at the last position (A). Screening the library against the two hairpins using the FID assay revealed that the mixture containing the pyrrole subunit at both the second (B) and third (C) positions gave the largest decrease in fluorescence with the PSA-ARE-3 sequence which contains a 5-bp GC bp at the center of the 5-bp A/T site. The affinity dropped for the ARE-consensus containing a GC bp at the center of the 5-bp A/T site of this sequence. Deconvolution by screening individual compounds of this mixture afforded the distamycin analogue (5) as having the highest affinity, followed closely by an analogue containing a thiophene subunit at the position A (Figure 9). Both compounds exhibited diminished affinity for the ARE-consensus sequence resulting from the intervening GC bp.33,49

Two other 10-compound mixtures also bound the PSA-ARE-3 sequence effectively. Deconvolution to individual compounds identified 6 and 7 as tight binders comparable to 5. Notably, 6 showed a loss of affinity for the ARE-consensus analogous to 5, but 7 retained equal affinity, making it ideal for binding both the PSA-ARE-3 and ARE-consensus sequences (Figure 9). Impressively, 7 exhibited potent (IC₅₀ = 8 nM) and selective (ca. 40-fold) inhibition of androgen receptor-mediated gene transcription in a cell-based reporter assay, albeit requiring liposome delivery of the compound for cell penetration and observation of activity.
Establishing Subtle Modes of Binding: Hairpin versus Extended Binding of Polyamides

The linkage of polyamides with \( \gamma \)-aminobutyric acid (\( \gamma \)) provides hairpin-bound polyamides that mimic the 2:1 side-by-side antiparallel binding of unlinked polyamides, enhances the binding affinity 10^2–10^4-fold, and improves the binding selectivity. In contrast, polyamides incorporating a one-carbon shorter head-to-tail linker, \( \beta \)-alanine (\( \beta \)), bind in an extended conformation, forming 1:1 or side-by-side antiparallel 2:1 complexes. A variant of the FID titrations was used to study the DNA binding properties of \( \alpha \)-substituted \( \beta \)-alanine-linked polyamides and a series of novel iminodiacetic acid (IDA)-linked polyamides (Figure 10). In these two series, the bound conformation (hairpin versus extended binding and parallel versus antiparallel binding) could be established by analysis of FID titrations of hairpin deoxyoligonucleotides containing a systematically varied A/T binding site size. Complementary assessments using a combination of footprinting and affinity cleavage techniques are technically more demanding, require the separate preparation of the linked Fe–EDTA affinity cleavage derivatives, and do not as easily distinguish between such alternative binding modes.

In the first of the two studies, a series of Py polyamides were linked using \( \alpha \)-substituted \( \beta \)-alanines to probe their impact on binding. Polyamide binding was assessed by using a series of hairpin deoxyoligonucleotides containing a systematically varied length of the A/T binding site (5–12 bp). The (R)-OMe-substituted derivative \( 9 \) bound with moderately high affinity, whereas the parent \( \beta \)-alanine \( 8 \) bound with intermediate affinity to the shortest hairpin (Figure 10). Very little change in the behaviors of \( 8 \) and \( 9 \) was seen until the length of the binding site reached 8 bp, where \( 8 \) dramatically changed. The binding constant increased by 2 orders of magnitude, and the stoichiometry of binding increased to 2. This corresponds to the expected behavior of extended binding over a 8–9-bp site as an antiparallel 2:1 side-by-side dimer. In contrast, the binding stoichiometry of \( 9 \) remained constant at 1:1, displaying a binding constant of \( \approx 8 \times 10^7 \text{M}^{-1} \) throughout the range of 5–10 bp until a binding site length of 11 bp was reached, where a second binding event was observed. The behavior seen with the 11- and 12-bp A/T sites represents two sequential binding events of \( 9 \) adopting a hairpin conformation, each requiring nonoverlapping 5-bp A/T sites (Figure 10).

The studies illustrated that a generalizable variant of the FID titrations may be utilized to distinguish hairpin versus extended binding, provide information on the...
binding site size and stoichiometry, and establish absolute affinity. Its use resulted in the discovery that, while most substituents on a \(\beta\)-alanine linker disrupt DNA binding favoring an extended binding mode, \((R)\)-\(R\)-methoxy-\(\beta\)-alanine maintains strong binding affinity and prefentially adopts a hairpin versus extended binding mode.51

In a second disclosure, the DNA binding properties of imidodiacetic acid-linked polyamides were examined (Figure 10). Utilizing the same series of hairpin deoxyoligonucleotides containing a systematically varied A/T binding site size, the binding affinity, stoichiometry, and binding site size were determined. Collectively it was determined that the IDA linker has a unique effect on the ability of the polyamides to bind DNA, providing what appears to be the first well-characterized example of cooperative parallel extended 2:1 side-by-side binding.52

DNA Binding Properties of Proteins

The DNA binding affinity and selectivity of proteins27 are typically assessed by selection screening,28–30 footprinting and affinity cleavage,14 or EMSA.31 Each technique possesses unique strengths, and the FID assay presents a complementary, technically non-demanding method for qualitative or quantitative assessment of DNA binding with concurrent establishment of the stoichiometry of binding.

Lymphoid enhancer-binding factor56 (LEF-1, also referred to as TCF-157,58) and closely related T-cell factors (TCF-159) are cell type-specific DNA binding proteins that play important regulatory roles.60,61 The majority of colorectal tumors contain mutations that result in accumulation of \(\beta\)-catenin. \(\beta\)-Catenin binds to and activates transcription factors including LEF-1, which binds the DNA minor groove through a high-mobility-group (HMG) domain, recognizing a consensus sequence \(5'\)CTTTGWW(W) \(3'\).56–63 A recent NMR structure of the complex of the LEF-1 HMG domain and a 15-bp deoxyoligonucleotide revealed that the protein makes extensive and continuous contacts in the DNA minor groove, encompassing the entire region implicated by chemical footprinting and mutagenesis.56–63

FID titration of the HMG domain of LEF-1 against a hairpin deoxyoligonucleotide containing \(5'\)CTTTGAAG provided a well-defined titration curve (Figure 11). Scatchard analysis provided a \(k_a\) of \(1.5 \times 10^9\) M\(^{-1}\), virtually identical to that established by EMSA.63 Once the viability of monitoring protein–DNA binding with the FID assay was confirmed, several other sequences were rapidly evaluated, including those implicated in initial studies, but not subsequently examined in detail. The third base of the consensus sequence, \(5'\)CTTTGWW, was permuted to each base variation and revealed that the C, G, and A substitutions bind the LEF-1 HMG domain effectively and almost indistinguishably, exhibiting \(k_a\)'s less than 2-fold lower than that of the reported consensus sequence (Table 4). As such, the consensus sequence of \(5'\)CTTTGWW is accurate but could easily by refined to \(5'\)CTNTGWW (N = G, C, A or T) to reflect this nearly indiscriminant third site.65

Although not demonstrated in this work, the FID assay could easily be extended to the examination of a library of proteins or mutants against such hairpins.

Summary and Outlook

The fluorescent intercalator displacement (FID) assay is a rapid, high-resolution, and technically non-demanding technique for establishing DNA binding selectivity and affinity for small molecules, proteins, and oligonucleotides.66 In a 96-well format, the assay provides for the high-throughput evaluation of a single compound against a library of DNA sequences (establish sequence selectivity) or for the high-throughput selection of high-affinity binders for a defined sequence from a library of compounds.
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Alternatively, FID titrations provide detailed information on single compounds and their binding to individual sequences, including binding constants, stoichiometry of binding, and binding site size. This latter application, as well as the selection screening against a single sequence, is amenable to examination of any sequence length. Its use in screening against a complete library of individual sequences has been exemplified with sequence sizes of ≤5-bp variable regions requiring a library of up to 512 hairpin deoxyoligonucleotides. Longer sequences require increasingly larger numbers of hairpins (e.g., 6 bp = 1024, 7 bp = 2048) or a pooling strategy for simple laboratory implementation. Unlike complementary techniques, the FID assay is nondestructive, providing the opportunity for hairpin immobilization onto reusable supports (chips, beads, or glass slides), thus removing the barrier to comprehensive and repeated screening of sequences longer than that presently exemplified.

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Table 4. HMG/LEF-1 Binding Constants

<table>
<thead>
<tr>
<th>DNA sequence</th>
<th>$K_a$ (M$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td>5′-CTTTGAAG$^a$</td>
<td>1.5 × 10$^9$</td>
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<tr>
<td>5′-CTTGTGTT</td>
<td>1.0 × 10$^9$</td>
</tr>
<tr>
<td>5′-CCCCCGAAG</td>
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<td>5′-CCTGGAAG</td>
<td>9.4 × 10$^8$</td>
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<tr>
<td>5′-CTATGGAAG</td>
<td>8.3 × 10$^8$</td>
</tr>
</tbody>
</table>

$^a$ Lit.$^{63} K_a = 1.0 × 10^{-9}$ M, EMSA.

References

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(26) Chodosh, L. A.; Carthew, R. W.; Sharp, P. A. A single polypeptide


(57) For a complete list of references, see ref 32, footnote 9.


(67) AR030113Y