CHAPTER 4
DNA-Protein Binding

DNA-Protein Binding

Introduction

Protein binding to DNA plays a fundamental role in regulating cellular and viral functions. The mechanisms by which proteins and DNA interact to control transcription and replication are slowly being elucidated. DNA-protein interactions are studied using a variety of methods such as gel-shift assays, footprinting, and transcriptional activation. While each of these methods may contribute distinct information about the location or effect of binding, they do not provide a simple way of quantitatively measuring specific binding. Fluorescence polarization/anisotropy provides a rapid, non-radioactive method for accurately measuring DNA-protein binding directly in solution without using filter binding, electrophoresis, or precipitation steps (Guest *et al.,* 1991; Heyduk and Lee, 1990; LeTilly and Royer, 1993; Lundblad *et al.,* 1996; Royer *et al.,* 1992).

In a basic fluorescence polarization experiment, a binding protein is serially diluted into a multiwell plate or disposable tubes. Fluorescein-labeled DNA containing the specific protein binding site is then added to each well or tube. After allowing the binding to reach equilibrium, the fluorescence polarization value of each sample is measured and the data points are used to construct an equilibrium binding curve. Since measuring polarization does not destroy the sample and because each reading takes less than 15 seconds, the polarization values of the samples can be repeatedly measured at different times or temperatures or both. The method is fast, simple and well-suited for optimizing binding conditions involving changes in buffers, detergents and DNA sequences, or the addition of non-specific proteins and/or nucleic acids.

• Guest, C.R. *et al.* (1991) *Biochemistry* **30:**8759-70.

• Heyduk, T., and Lee, J.C. (1990) *Proc. Natl. Acad. Sci. USA.* **87:**1744-8.

• LeTilly, V., and Royer, C.A. (1993) *Biochemistry* 32:7753-8.

• Lundblad, J.R. *et al.* (1996) *Mol. Endocrinol.* **10:**607-12.

• Royer, C.A. *et al.* (1992) *Anal. Biochem.* **191:**287-94.

Binding of Human Recombinant Estrogen Receptor to a Fluorescein-labeled Estrogen Response Element (U.S. Patent No. 5,445,935)

Steroid hormone receptors play a vital role in regulating cellular growth and differentiation. This conserved superfamily of proteins binds many classes of steroid hormones including estrogens, progesterones, glucocorticoids, and androgens. Though diverse in the biological functions that they control, these protein receptors are generally conserved in four domains: the N-terminus, a less-conserved hinge region, a hormone binding domain, and a well-conserved DNA binding domain. When the cytosolic estrogen receptor binds hormone, the complex moves into the nucleus where it acts as a transcription factor, binding to estrogen responsive elements (ERE) in the DNA and thereby modulating a myriad of cellular functions. Fluorescence anisotropy was used to study the equilibrium binding between the human estrogen receptor (hER) and a double-stranded, fluorescein-labeled oligonucleotide containing an estrogen response element (ERE-F). The general scheme is shown in **Figure 4-1**:

Fluorescein-labeled ERE

hER

Low Anisotropy

Fluorescein-labeled ERE:hER Complex

hER

High Anisotropy

Figure 4-1. Principle of the F-ERE:hER binding assay. When measuring the polarization of ERE-F in the absence of hER, the labeled DNA is free to rotate in solution (low polarization value). When the ERE-F is bound by hER, the large complex is slow to rotate in solution (high polarization value).

Both DNA strands were synthesized and labeled with fluorescein attached via a six-carbon spacer at the 5' terminus. The 50 base-pair, double-stranded oligonucleotide (ERE-F):

ERE1-F: 5'-**F**-CGATCAGATTAGAGCTGATGCCTCCGGTCACTGTGACCCAACCCATCTTA-3'

ERE2-F: 3'-GCTAGTCTAATCTCGACTACGGAGGCCAGTGACACTGGGTTGGGTAGAAT-**F**-5'

was prepared by annealing equimolar concentrations of each strand in 1 M NaCl, 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0). This mixture was heated to 95°C for 10 minutes and slowly cooled (30 minutes) to room temperature.

The hER was serially diluted in TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) from 200 to 0.1 nM in 12 reaction tubes with a final volume of 100 µL. ERE-F was added to each tube to a concentration of 1 nM and the binding reactions were incubated at room temperature for 30 minutes. The fluorescence anisotropy of each tube was measured using the Beacon® 2000 Fluorescence Polarization System. The equilibrium binding data were analyzed using non-linear regression and plotted (**Figure 4-2**). At hER concentrations below 1 nM, the ERE-F remains free and has a low anisotropy value (mA = 50). As the hER concentration increases, a greater fraction of the fluorescein-labeled oligonucleotide is bound and the anisotropy progressively increases to a maximum value of 200 mA. The K_d (the hER concentration at which 50% of the ERE-F is bound) in this experiment was 4.5 ± 1.0 nM.

Figure 4-2. Binding of Estrogen Receptor (hER) to a Fluorescein-labeled Estrogen Response Element (ERE-F). hER was serially diluted in 10 mM Tris-HCl, 1 mM EDTA (pH 7.5), from 200 to 0.1 nM in 100 µL reaction tubes. ERE-F was added to each tube to a concentration of 1 nM. Fluorescence anisotropy was measured using the Beacon® 2000 Fluorescence Polarization System. Actual data points and the computer-fitted curve are shown. The K_d for this interaction was found to be 4.5 ± 1.0 nM.
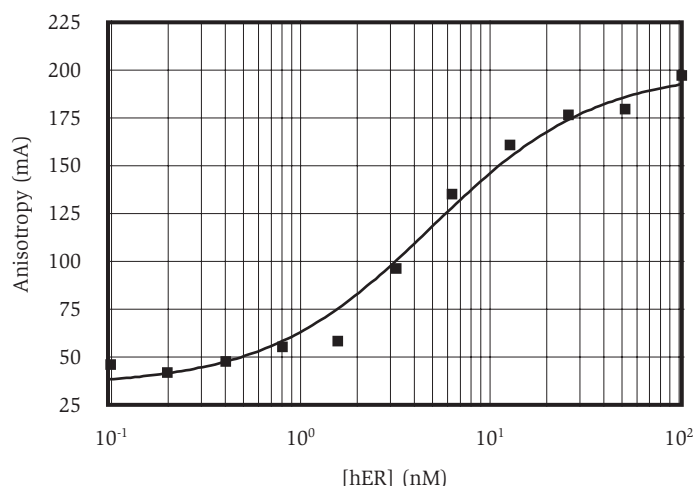
Binding of *trp* Repressor to a Fluorescein-labeled *trp* Repressor Operator (U.S. Patent No. 5,445,935)

The *trp* repressor, when bound to its co-repressor tryptophan, binds tightly to a specific DNA sequence in the operator region of the *trp* EDCBA operon in the *E. coli* genome. When bound to the operator, the *trp* repressor suppresses transcription of the genes involved in tryptophan biosynthesis. Binding between the *trp* repressor and the operator has been studied using traditional filter binding and gel retardation techniques (Carey, 1988). LeTilly and Royer (1993) showed that fluorescence anisotropy provides a simple, direct method for measuring the equilibrium binding between *trp* repressor and its operator DNA. In the example presented here, equilibrium binding of the *trp* repressor (TrpR) to a fluorescein-labeled oligonucleotide (*trp*O-F) containing the *trp* operator was measured. As increasing amounts of protein bind to the labeled DNA, the molecular rotation of the oligonucleotide decreases, leading to an increase in the fluorescence anisotropy of the reaction mixture. This is illustrated in **Figure 4-3**:

Figure 4-3. Diagram of TrpR binding to *trp*O-F in a FP-based Assay. In this example, the receptor binds to the labeled DNA (which initially had a low polarization value), creating a larger complex that has a high polarization value. T = tryptophan
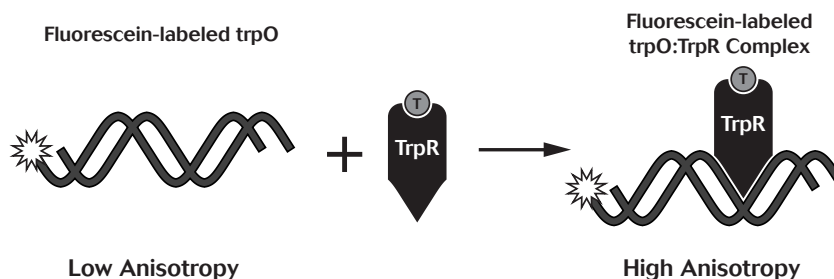
The oligonucleotides used for this experiment are shown below. The Trp25-F oligo was labeled with the fluorescein attached to its 5' end via a 6-carbon spacer:

Trp25-F: 5'-**F**-ATCGAACTAGTTAACTAGTACGCAA-3'

Trp25-A: 3'-TAGCTTGATCAATTGATCATGCGTT-5'

The 25 base-pair, double-stranded oligonucleotide (trpO-F) was prepared by annealing the sense and antisense strands in 1 M NaCl, 10 mM potassium phosphate, 0.1 mM EDTA (pH 7.6). The mixture was heated to 95°C for 10 minutes and slowly cooled to room temperature

• Carey, J. (1988) *Proc. Natl. Acad. Sci. USA* **85:**975-9.

• LeTilly, V., and Royer, C.A. (1993) *Biochemistry* **32:**7753-8.

over 30 minutes. The TrpR was serially diluted from 1 µM to 3.9 pM in 1.0 mL 10 mM potassium phosphate, 0.1 mM EDTA (pH 7.6), 4 mM tryptophan and 10% glycerol in 10×75 mm disposable borosilicate test tubes (Invitrogen Part No. P2245). The *trp*O-F was added to each tube, yielding a final concentration of 200 pM. The anisotropy of each reaction tube was measured using the Beacon® 2000 Fluorescence Polarization System and is shown in **Figure 4-4**. These equilibrium binding data were obtained under conditions similar to those used previously in a gel retardation assay (Carey, 1988). At low TrpR concentrations (less than 0.1 nM), the *trp*O-F is free and has a low anisotropy (mA = 70). As the trp repressor concentration increases, a greater fraction of the *trp*O-F is bound in the DNA-protein complex and the anisotropy increases to a maximum of 200 mA.
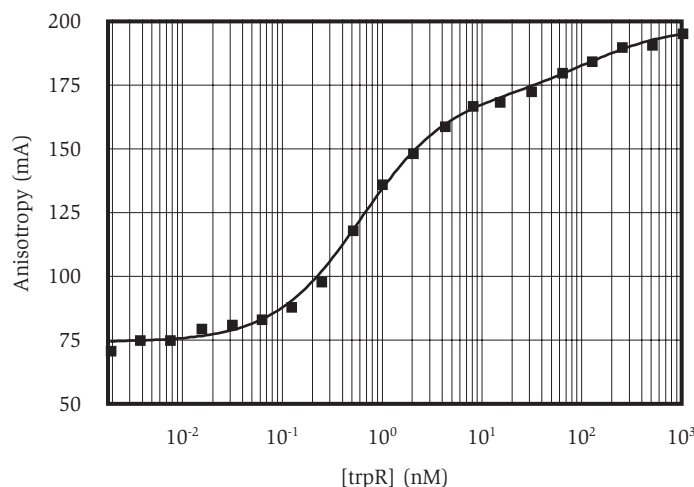

Figure 4-4. Trp Repressor Binding to *trp*O-F. Recombinant Trp repressor, purified from *E. coli,* was serially diluted from 1 µM to 3.9 pM in 10 x 75 mm tubes containing 10 mM potassium phosphate, 0.1 mM EDTA (pH 7.6), 4 mM tryptophan, 10% glycerol. *trp*O-F was added to each tube to a concentration of 200 pM. Fluorescence anisotropy was measured using the Beacon® 2000 Fluorescence Polarization System.

The *trp* repressor/operator equilibrium binding data could not be fitted to a simple sigmoidal curve, suggesting the presence of multiple binding equilibria, which was predicted by LeTilly and Royer (1993) as they characterized the complex *trp* repressor monomer-dimer and dimer-tetramer DNA equilibria. Please refer to this reference for a detailed description of the analysis of this complex binding isotherm. The large number of high quality data points obtained permits the analysis of complex, multiple DNA/protein binding equilibria.

- Carey, J. (1988) *Proc. Natl. Acad. Sci. USA* **85:**975-9.

- LeTilly, V., and Royer, C.A. (1993) *Biochemistry* **32:**7753-8.

Binding of Nuclear Factor-IL6 to the
Rat Angiotensinogen Promoter (U.S. Patent No. 5,445,935)

Nuclear factor-IL6 (NF-IL6) is a member of the CCAAT-box/Enhancer Binding Protein family and contains a basic domain DNA binding motif. NF-IL6 attenuates the activity of the acute-phase response element (APRE) of the angiotensinogen gene by displacing the NF-κB transactivator from an overlapping binding site (Brasier *et al.*, 1990).

In this study, the equilibrium dissociation constants (K_d) were determined for the binding of two truncated NF-IL6 peptides to a fluorescein-labeled, doubled-stranded DNA oligonucleotide corresponding to the rat angiotensinogen APRE (APRE M6-F). The first peptide, called the tryptic core domain (TCD), was produced from trypsin digestion of NF-IL6-DNA complexes and corresponds to residues 266-345. The second peptide, D^{14}, produced from Endoprotease Asp-N cleavage of NF-IL6, corresponds to residues 272-345.

The peptides were serially diluted into 20 mM Tris-HCl (pH 7.6), 100 mM KCl, 0.1 mM EDTA and 1 mM DTT, creating 20 concentrations of each peptide in a volume of 1 mL. The D^{14} peptide was serially diluted from 25 µM to 48 pM and the TCD peptide was diluted from 80 µM to 153 pM. Fluorescein-labeled oligonucleotide was then added to each tube (10 µL of a 100 nM stock solution) for a final DNA concentration of 1 nM. The tubes were incubated at 25°C for 30 minutes before anisotropy values were determined. Equilibrium binding data were analyzed using non-linear regression.

The D^{14} peptide, containing residues 272-345, generated a binding curve that could be fit using a single-site model with a K_d of 262 nM (**Figure 4-5**). On the other hand, the TCD peptide, representing residues 266-345, was fit best by a two-site model with a high affinity site ($K_d = 46$ nM) and low affinity site ($K_d = 8400$ nM, **Figure 4-5**).

Figure 4-5. Binding Isotherm of TCD and D14 Peptide/DNA Interaction. Increasing amounts of TCD or D^{14} peptide were incubated with 1 nM APRE M6-F, a fluorescein-labeled 33 base-pair oligonucleotide corresponding to the rat angiotensinogen APRE, in 20 mM Tris-HCl (pH 7.6), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT. The tubes were incubated for 30 minutes at 25°C before fluorescence anisotropy values were determined.
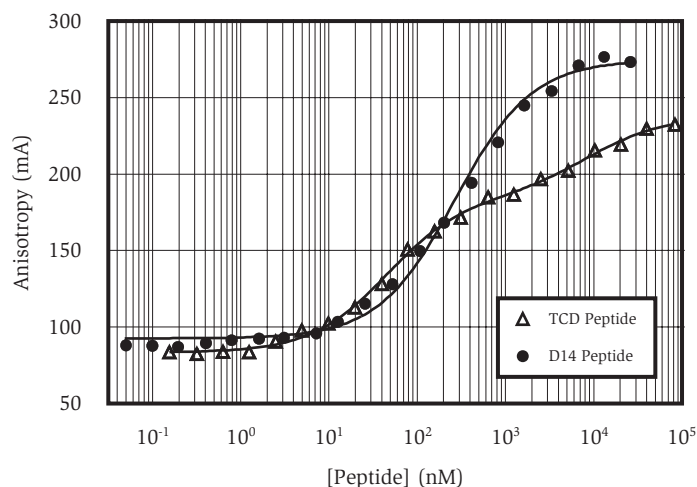
These data are in close agreement with the work of Brasier and Kumar (1994) who estimated the K_d for the D^{14} peptide-DNA binding as 283 nM and the K_d for TCD peptide-DNA binding as 36 nM using a gel mobility shift assay. Analysis of the gel-shift data did not identify the TCD low-affinity binding site, presumably because of the limited number of data points associated with this technique. The authors speculate that the NF-IL6 complex stabilizing domain, residues 262-272, is responsible for the tight binding of NF-IL6 to rat angiotensinogen APRE (Brasier and Kumar, 1994).

• Brasier, A.R. *et al.* (1990) *EMBO J.* **9:**3933-44.

Temperature-dependent Binding of TATA Binding Protein (TBP) to the TATA Box Consensus Sequence (U.S. Patent No. 5,445,935)

The TATA box is the most well-known promoter element in eukaryotes. The majority of eukaryotic genes contain this sequence upstream from the transcription start site. The TATA box serves as an anchor for the formation of transcription pre-initiation complexes to which RNA polymerase binds. Binding of the TATA Binding Protein (TBP) to the TATA box is the first step in the formation of the transcription pre-initiation complex (Nakajima *et al.*,1988). TBP demonstrates DNA binding properties unique among the sequence-specific DNA binding proteins, namely a strong temperature dependence of binding and slow on/off rates (Horikoshi *et al.*, 1988; Lee *et al.*, 1992).

In this study, the temperature-dependent binding of yeast TBP (yTBP) to the TATA box consensus sequence from adenovirus major late promoter is characterized. Equilibrium dissociation constants (K_d) were determined for yTBP binding to the TATA box at both 25°C and 37°C. The TBP peptide was diluted serially (1:1.5) into 20 mM Tris-HCl (pH 7.8), 80 mM KCl, 10 mM $MgCl_2$, 0.2 mM EDTA and 1 mM DTT using Beacon® 12×75 mm borosilicate test tubes (Invitrogen Part No. P2182). TBP concentrations ranged from 4.4 μM to 889 pM (22 samples with a volume of 1 mL). The double-stranded, fluorescein-labeled TATA box oligonucleotide was added to each tube (10 μL of 100 nM stock solution) for a final DNA concentration of 1 nM. The tubes were incubated at 25°C for 30 minutes after which the anisotropy values were measured. The same tubes were then incubated for 30 minutes in a 37°C water bath and the anisotropy values were measured again. At 25°C, these data did not fit a simple binding model, so it was not possible to estimate (with certainty) the K_d for the low affinity binding of TBP to the fluorescein-labeled TATA box (see **Figure 4–6**). After reincubating the sample tubes at 37°C for 30 minutes, a high-affinity binding curve emerged with a K_d of 60 nM. This work is consistent with the findings of Lee *et al.* (1992) who demonstrated by gel mobility shift assay that TBP does not bind to the TATA box at 4°C, but does bind tightly at 30°C. Fluorescence anisotropy has proven to be particularly well-suited for this type of temperature-dependent binding study. Taking these measurements is non-destructive, allowing the researcher to measure binding events in the same tube or multiwell plate under a variety of conditions.

- Brasier, A.R. and Kumar, A. (1994) *J. Biol. Chem.* **269:**10341-51.

- Nakajima, N. *et al.* (1988) *Mol. Cell. Biol.* **8:**4028-40.

- Horikoshi, M. *et al.* (1988) *Cell* **54:**1033-42.

- Lee, D.K. *et al.* (1992) *Mol. Cell. Biol.* **12:**5189-96.

Figure 4-6. Binding Isotherm of TBP to TATA Box Consensus Sequence. Various concentrations of recombinant yTBP purified from *E. coli* were incubated with 1 nM fluoresceinated TATA box DNA corresponding to bases -52 to +10 of the adenovirus major late promoter sequence at 25°C (squares) for 30 minutes in 20 mM Tris-HCl (pH 7.8), 80 mM KCl, 10 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT. Fluorescence anisotropy was measured in each tube. Then, the same tubes were incubated at 37°C (triangles) for 30 minutes and fluorescence anisotropy values were measured again. High-affinity binding was observed only at the elevated temperature of 37°C.
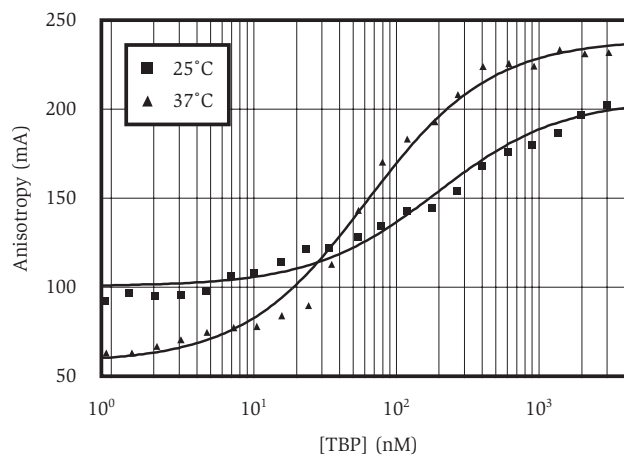
Acknowledgment

Considerations for DNA-Protein Binding Studies

How much protein is required to measure equilibrium binding to DNA? The amount of protein required depends on the molecular weight of that protein and how tightly it binds DNA. Use the following example as a guideline. Assume a 35,000 Da protein binds to DNA with a K_d of 2.5 nM. The protein is serially diluted (1.5-fold for each dilution) from 250 nM to 0.11 nM in 18 tubes. Then, 250 pM fluorescein-labeled DNA is added to each tube and binding is allowed to reach equilibrium. Fluorescence polarization values are then determined for each tube. **Table 4-1** lists the amount of protein in each tube and shows that the total protein required for 18 data points is 26.2 µg. If the protein bound more tightly with a K_d of 0.25 nM, then the experiment would require 2.62 µg of protein. If the protein had a molecular weight of 17,500 Da, the experiment would require 13.1 µg of protein. Note that >90% of the protein is in the first six tubes.

Table 4-1. Theoretical amount of protein required to measure the equilibrium binding to DNA using 18 data-points. In this example, it was assumed that the protein had a M.W. of 35,000 Da and binds to the DNA with a K_d of 2.5 nM

Test Tube Number	Protein Concentration (nM)	Protein/Test Tube (ng)
1	250	8,750
2	167	5,833
3	111	3,889
4	74	2,593
5	49	1,728
6	33	1,152
7	22	768
8	15	512
9	10	341
10	6.5	228
11	4.3	152
12	2.9	101
13	1.9	67
14	1.3	45
15	0.9	30
16	0.6	20
17	0.38	13
18	0.25	9
		Total 26,232

An alternative method is to use a single tube with 200 pM labeled DNA and incrementally add protein. The fluorescence polarization would be measured after each protein addition. For the 35 kDa protein with a K_d of 2.5 nM, 8.75 µg of protein would be required, theoretically. Considering losses upon dilution, however, 10-15 µg is more realistic. This protocol is more time-consuming than the serial-dilution method and is more likely to introduce pipetting errors, but it does minimize the amount of protein used. Optimization of equilibrium binding conditions is described in detail in **Chapter 8.**

What should the size of my labeled DNA be? We have successfully tested 5' end-labeled DNA from 20 to 150 base pairs. DNA with sizes outside this range may also work successfully.

What is the best way to label the DNA? Generally, we suggest having oligonucleotides chemically synthesized with the fluorescein attached. If larger DNA molecules are required, labeled nucleotides or oligos can be used in the polymerase chain reaction to produce large amounts of labeled DNA. Oligonucleotides can be purchased with a 5' amino linker added as a phosphoramidite during synthesis. The oligonucleotide can then be specifically labeled at the primary amine using succidimidyl esters of common fluorophores such as fluorescein or rhodamine.

Can I use labels other than fluorescein on the Beacon® 2000 Fluorescence Polarization System? The Beacon® 2000 System comes standard with filters suitable for fluorescein. Because the holders allow easy filter exchange, the Beacon® 2000 Analyzer can be used with any fluorophore that is excitable by the halogen light source and that fluoresces at a wavelength detectable by the photomultiplier tube (360 to > 700 nm). Consult Invitrogen for custom filter sets to be used with fluorophores other than fluorescein.

How long does it take to develop a binding curve for my protein? This depends on how fast the binding equilibrates. Binding reaches equilibrium very quickly (within a few minutes) for *trp* repressor, for example. Other systems may require more time to reach equilibrium. We suggest determining the time required to approach equilibrium for each system you study, as you would for any equilibrium binding technique. This can be accomplished by measuring the time-dependent binding of a single tube (usually one containing the lowest concentration of reagents) on the Beacon® 2000 System in Kinetic Mode.

Will this system work for RNA as well as DNA? Yes. Keep in mind that to maximize the shift in polarization upon binding, one needs to design the system so that there is a maximum change in the mobility of the fluorescent label upon binding. An RNA binding protein binding to fluorescein-labeled RNA should work just as well as a DNA/protein system. The proximity of the binding site to the fluorescent label can be critical to maximize the shift in polarization upon binding.

My protein binds best at 42°C. Can I still use the Beacon® 2000 Fluorescence Polarization system? Yes. The Beacon® 2000 operates with the sample chamber at temperatures from less than 6°C to greater than 65°C, adjustable in 1°C increments.

Will fluorescence polarization replace all my gel-shift assays? No. As demonstrated in **Table 4-1**, significant amounts of protein may be required for some experiments. While partially purified proteins have been studied using fluorescence polarization, it is best to work with abundant sources (clones) of DNA binding proteins.