Technical Resource Guide FOURTH EDITION

Fluorescence Polarization

invitrogen™

Contents

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Patents

The method for detecting nucleic acid cleavage utilizing a fluorescent tag in the Beacon® 2000 RNase Activity Detection Kit and the Beacon® 2000 DNase Activity Detection Kit is covered by U.S. Patent No. 5,786,139. Other U.S. and international patents pending.

The method for detecting reverse transcriptase activity using fluorescence polarization is covered by U.S. Patent No. 6,100,039. Other U.S. and international patents pending.

FOREWORD
to the Fourth Edition

The theory of fluorescence polarization (FP) was first described by Jean Perrin in 1926 and expanded by Gregorio Weber and others in the 1950's. Surprisingly, even into the late 1980s this powerful technique was almost unknown to all but the diagnostic industry and biophysicists. Typically, the only exposure biochemists and molecular biologists have had to FP is a single chapter in Joseph Lakowicz's seminal work, "Principles of Fluorescence Spectroscopy".

In 1993, we set out to investigate FP for the study of biomolecular interactions and develop it has a core technology for PanVera. Unfortunately, there were no instruments available that could measure FP easily. Typical FP instruments were hand-built from analytical fluorescence spectrophotometers and often required manual operation.

Realizing the need for an easy-to-use, sensitive, bench-top FP instrument we began marketing the Beacon® instrument in 1994. Two years later, we introduced the Beacon® 2000, which boasted better sensitivity than laser-based analytical instruments, used disposable glass test tubes, temperature controls, and a minimum volume requirement of only 100 μL.

PanVera published the First Edition of the Fluorescence Polarization Application Guide in 1995. It was filled with data generated on the Beacon® 2000 and how-to information. We knew that until FP was demystified for the at-large scientific community, the technology's full potential would not be realized. With this guide, it was our intention to empirically demonstrate the versatile nature of FP and how it could be used to observe a wide range of biomolecular interactions. FP differed dramatically from all other methods in use at that time in that it was a truly homogeneous technique, required no separation of bound and free species, no radioactivity, and allowed real-time measurements to be made directly in solution. We also highlighted some of the differences one must consider when designing FP-based assays. For example, FP differs from a traditional radioactive binding assay in that the small fluorescent ligand is held at a low concentration while the larger binding partner is titrated into it. Therefore, basic binding equations had to be discussed and modified slightly to accommodate these differences.

It did not take long for researchers in drug discovery to realize that FP is a format well suited for high throughput screening (HTS). Instrumentation is now available that can measure FP in high-density microplates very rapidly and with great precision. Assays require very few additions and no separation steps. No immobilization of reaction components is required, reducing the potential for artifacts generated by attaching molecules to solid supports. The method is non-radioactive, improving safety and reducing the costs associated with waste disposal. We continue to build on our extensive knowledge base and long history in this field to produce innovative assays for drug discovery.

It is our hope that the Fourth Edition of this Guide will help the novice gain a basic understanding of FP while serving as a desktop reference to the initiate.

CHAPTER 1
Introduction

Introduction

Fluorescence polarization (FP) is a powerful tool for studying molecular interactions by monitoring changes in the apparent size of fluorescently-labeled or inherently fluorescent molecules, often referred to as the tracer or ligand (Checovich *et al.,* 1995; Heyduk *et al.,* 1996; Jameson and Sawyer, 1995; Nasir and Jolley, 1999). It is unique among methods used to analyze molecular binding because it gives a direct, nearly instantaneous measure of a tracer's bound/free ratio.

FP enables the researcher to view molecular binding events in solution, allowing true equilibrium analysis into the low picomolar range (*i.e.,* with as little as 10 fmol/mL of sample stoichiometrically labeled with fluorescein). FP measurements do not affect samples, so they can be treated and reanalyzed in order to ascertain the effect on binding by such changes as pH, temperature, and salt concentration. In addition, because FP measurements are taken in "real-time," experiments are not limited to equilibrium binding studies. Kinetic analysis of association and dissociation reactions are routine with fluorescence polarization.

Because FP is a truly homogeneous technique, it does not require the separation of bound and free species. Methods that depend on separation are not only more time-consuming, but they disturb the reaction equilibrium and therefore prevent accurate quantification of binding. Alternative homogeneous fluorescent techniques, such as fluorescence resonance energy transfer (FRET) and time-resolved fluorescence or TR-FRET (Pope *et al.,* 1999) require multiple labeling reactions instead of one as in FP.

Theory

First described by Perrin (1926), the theory of FP is based on the observation that when a small fluorescent molecule is excited with plane-polarized light, the emitted light is largely depolarized because molecules tumble rapidly in solution during its fluorescence lifetime (the time between excitation and emission). However, if the tracer is bound by a larger molecule its effective molecular volume is increased. The tracer's rotation is slowed so that the emitted light is in the same plane as the excitation energy. The bound and free states of the tracer each have an intrinsic polarization value: a high value for the bound state and a low value for the free state. The measured polarization is a weighted average of the two values, thus providing a direct measure of the fraction of tracer bound to receptor. An increase in molecular volume due to receptor-ligand (Bolger *et al.,* 1998), DNA-protein (Lundblad *et al.,* 1996; Ozers *et al.,* 1997), or peptide-protein binding (Wu *et al.,* 1997) or a decrease in molecular volume due to dissociation or enzymatic degradation (Bolger and Checovich, 1994; Bolger and Thompson, 1994) can be followed by FP.

As illustrated in **Equation 1.1**, the polarization value of a molecule is proportional to the molecule's rotational relaxation time, or the time it takes to rotate through an angle of 68.5°. Rotational relaxation time is related to viscosity (η), absolute temperature (T), molecular volume (V), and the gas constant (R).

$$\text{Equation 1.1: Polarization value} \propto \text{Rotational relaxation time} = \frac{3\eta V}{RT}$$

- Bolger, R. and Checovich, W. (1994) *BioTechniques* **17:**585–9.

- Bolger, R. and Thompson, D. (1994) *Am. Biotechnol. Lab.* **12:**113–6.

- Bolger, R. *et al.* (1998) *Environ. Health Perspect.* **106:**551–7.

- Checovich, W. J. *et al.* (1995) *Nature* **375:**254–6.

- Heyduk, T. *et al.* (1996) *Meth. Enzymol.* **274:**492–503.

- Jameson, D.M. and Sawyer, W.H. (1995) *Meth. Enzymol.* **246:**283–300.

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

- Nasir, M.S. and Jolley, M.E. (1999) *Comb. Chem. High Throughput Screen.* **2:**177–90.

- Ozers, M.S. *et al.* (1997) *J. Biol. Chem.* **272:**30405–11.

- Perrin J. (1926) *Phys. Rad.* **1:**390–401.

- Pope, A.J. *et al.* (1999) *Drug Discovery Today* **4:**350–62.

- Wu, P. *et al.* (1997) *Anal. Biochem.* **249:**29–36.

Therefore, if viscosity and temperature are held constant, polarization is directly related to the molecular volume (*i.e.,* molecular size). Changes in molecular volume can result from binding or dissociation of two or more molecules, degradation, or from conformational changes.

Fluorescence polarization detection is described schematically in **Figure 1-1**. Monochromatic light passes through a vertical polarizing filter and excites fluorescent molecules in the sample tube. Only those molecules that are oriented properly in the vertically polarized plane absorb light, become excited, and subsequently emit light. The emitted light is measured in both the horizontal and vertical planes.
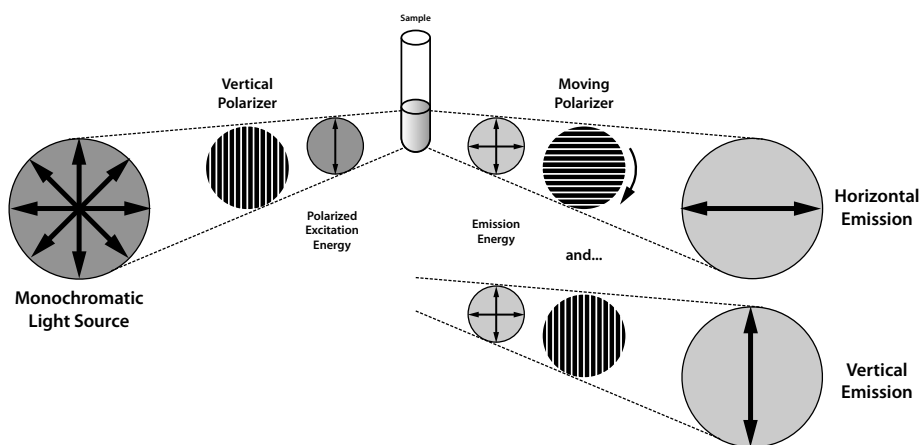
Figure 1-1. Schematic representation of FP detection. Monochromatic light passes through a vertical polarizing filter and excites fluorescent molecules in the sample tube. Only those molecules that are oriented properly in the vertically polarized plane absorb light, become excited, and subsequently emit light. The emitted light is measured in both the horizontal and vertical planes.

Polarization is calculated as shown in **Equation 1.2** and is a measure of the extent of molecular rotation during the period between excitation and emission.

$$\text{Equation 1.2: Polarization value (P)} = \frac{\text{Intensity}_{vertical}\ 2\ \text{Intensity}_{horizontal}}{\text{Intensity}_{vertical}\ 1\ \text{Intensity}_{horizontal}}$$

The polarization value, P, being a ratio of light intensities, is a dimensionless number, often expressed in millipolarization units (1 Polarization Unit = 1000 mP Units).

Illustrated examples of complexes with high and low polarization values are shown in **Figure 1-2**. Small molecules rotate quickly during the excited state, and upon emission, have low polarization values. Large molecules, in this case caused by the binding of a second molecule, rotate little during the excited state, and therefore have high polarization values.
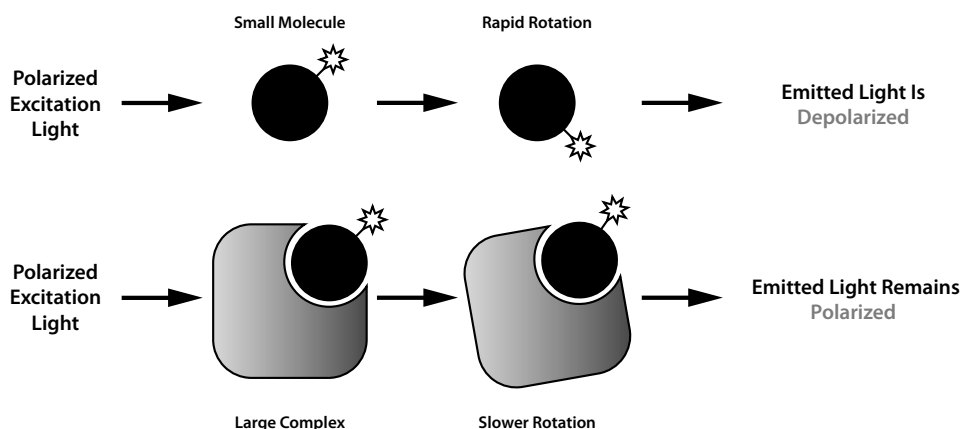
Figure 1-2. Schematic of FP differences between small and large complexes. Small molecules rotate quickly during the excited state, and upon emission, have low polarization values. Large molecules, in this case caused by the binding of a second molecule, rotate little during the excited state, and therefore have high polarization values.

Anisotropy or Polarization?

The term "anisotropy" is sometimes used in literature associated with the FP field. Polarization and anisotropy are both derived from the measured vertical and horizontal intensities. The values are mathematically related and easily interconverted. Both values represent a weighted average of the bound versus unbound states of the fluorescent molecule.

While anisotropy and polarization share the same content of information, anisotropy values are mathematically easier to manipulate in many FP studies. A population of excited, identical molecules in solution will all have the same polarization value. If a portion of these molecules undergoes an apparent size change such that their rotational relaxation rate also changes, the observed polarization value represents an average of the component polarizations of all of the molecules. The polarization value of a mixture of molecules was described by Weber (1952) and is shown here as **Equation 1.3**:

$$\text{Equation 1.3:} \qquad \left(\frac{1}{P} - \frac{1}{3} \right)^{-1} = \sum_{i=1}^{n} f_i \left(\frac{1}{P_i} - \frac{1}{3} \right)^{-1}$$

where each fluorophore species has a polarization value (P_i) and fractional fluorescence intensity (f_i). On the other hand, the additivity of anisotropies is given by a simpler equation:

$$\text{Equation 1.4:} \qquad A = \sum_{i=1}^{n} f_i \times A_i$$

Due to mathematical simplicity, anisotropy values are sometimes preferred because it is easier to deconvolute anisotropy values into their component values than it is with polarization values. It should be noted again that in the majority of applications, anisotropy does not give any additional information over polarization. The issue of using anisotropy versus polarization is discussed further in **Chapter 8**.

We generally use the term "Fluorescence Polarization" instead of "Fluorescence Anisotropy" because FP is most often the term used to describe the entire technology. In many applications that involve a minimum of curve analysis, we use polarization out of habit and tradition and because the error involved when using polarization instead of anisotropy is nominal.

• Weber, G. (1952) *Biochem*. **51**:145-55.

The Beacon® 2000 Fluorescence Polarization System

Historically, fluorescence polarization had been used worldwide in the human diagnostic market for more than a decade (Jolley, 1981), but it had seen limited application in the broader research market due to the lack of sensitive, versatile, and affordable instrumentation. Invitrogen developed one of the original instruments that met all of these requirements—the Beacon® 2000 Fluorescence Polarization System (Invitrogen Part No. P2300). The Beacon® 2000 System, which is a single-tube instrument, continues to provide a rapid and reliable way to measure equilibrium binding or molecular degradation for a wide range of biological molecules including proteins, nucleic acids, carbohydrates, lipids, and drugs. The Beacon® 2000 Instrument was designed specifically to meet research needs. It is a compact, benchtop instrument that takes measurements on samples *directly in solution* and provides *picomolar* sensitivity (*i.e.*, low fmol/mL of sample stoichiometrically labeled with fluorescein). Applications as varied as protein-DNA interactions, immunoassays, protease assays, epitope mapping, DNA hybridization, and receptor-ligand binding studies are easily performed on the Beacon® 2000 System.

Because FP is now routinely used in the high throughput screening environment, newer and faster multiwell plate-based instruments are often the tool of choice for these industrial applications.

High Throughput screening (HTS) using FP

The tremendous challenge facing high throughput screening (HTS) scientists today is to screen more compounds against more targets using more quantitative and robust methods without spending more money. Genomics efforts have flooded drug discovery with potential new drug targets (Drews, 1996). New parallel combinatorial synthesis methods are providing more compounds that must be screened for activity (Gallop *et al.*, 1994). The process for identifying new lead compounds must become more efficient. In order to increase the efficiency of HTS, new screening methods must be faster, cheaper, and more quantitative. Assays need to be miniaturized to decrease reagent costs and consumption of compound libraries. New assay formats must be homogeneous, requiring no separation of reaction components.

Fluorescent methods are rapidly becoming the primary detection format in HTS because they now approach the sensitivity of radioactive techniques and are amenable to homogeneous and miniaturized formats (Pope *et al.*, 1999). The increasing use of homogeneous fluorescence methods continues to be driven by a mandate within large pharmaceutical companies to significantly reduce the use of radioactivity in all facets of pharmaceutical research due to waste management issues. Homogeneous formats are desirable because these formats are "mix-and-read" without wash steps, multiple incubations, or separations required. Because FP measurements are made directly in solution, no perturbation of the sample is required, making the measurement faster and more quantitative than conventional methods.

• Drews, J. (1996) *Nat. Biotechnol.* **14**:1516-17.

• Gallop, M.A. *et al.* (1994) *J. Med. Chem.* **37**:1233-51.

• Jolley, M.E. (1981) *J. Anal. Toxicol.* 5:236-40.

• Pope, A.J. *et al.* (1999) *Drug Discovery Today* **4**:350-62.

Since 1995, there have been a number of publications, listed in **Table 1-1** (adapted from Owicki, 2000), detailing the development of HTS assays using FP. As these articles demonstrate, FP has been most applicable when the HTS assay involves measuring changes in the fraction-bound of a small, fluorescently-labeled molecule and a large, unlabeled molecule. The kinase assays are actually FPIAs (FP immunoassays) in which a phosphopeptide formed in a kinase reaction displaces a fluorescently-labeled phosphopeptide from a phospho-specific antibody. The nuclear receptor assays are ligand displacement assays where the affinity of test compounds for a receptor are measured by their ability to displace a bound fluorescent ligand. These and other assay types are described in detail later in this Guide.

Table 1-1. Publications detailing the development of HTS assays using FP.	
Publication	Assay Type/Target Class
Deshpande *et al.* (1999)	Kinase
Parker *et al.* (2000)	Kinase
Wu *et al.* (2000)	Kinase
Parker *et al.* (2000)	Phosphatase
Parker *et al.* (2000)	Nuclear Receptors
Keating *et al.* (2000)	Protein-Protein
Lynch *et al.* (1997)	Protein-Protein
Lynch *et al.* (1999)	Protein-Protein
Wu *et al.* (1997)	Protein-Protein
Jolley (1996)	Protease
Levine *et al.* (1997)	Protease
Pope *et al.* (1999)	Protease
Chen *et al.* (1999)	Genomics
Pope *et al.* (1999)	Nucleic Acid
Allen *et al.* (2000)	GPCR
Banks *et al.* (2000)	GPCR
Pope *et al.* (1999)	Topoisomerase
Pope *et al.* (1999)	Cytokine Receptors
Li *et al.* (2000)	Transferase
Zhao *et al.* (1999)	Antimicrobials
Kauvar *et al.* (1995)	Protein-fluorescent probe binding
Sportsman *et al.* (1997)	Protein-fluorescent probe binding

The purpose of this Guide is to give the reader an intuitive feel for the utility of fluorescence polarization. HTS scientists will continue to increase their use of FP due to its unique combination of simplicity, speed, and robustness. Additional improvements in multiwell instrumentation, and the discovery of longer-lifetime and longer-wavelength fluorophores will expand its use even further.

- Allen, M. *et al.* (2000) *J. Biomol. Screen.* **5**:63-70.
- Banks, P. *et al.* (2000) *J. Biomol. Screen.* **5**:159-68.
- Chen, X. *et al.* (1999) *Genome Res.* **9**:492-8.
- Deshpande, S.*et al.* (1999) *Prog. Biomed. Optics (SPIE)* **3603**:261-61.
- Jolley, M. (1996) *J. Biomol. Screen.* **1**:33-8.
- Kauvar, L.M. *et al.* (1995) *Chem. Biol.* **2**:107-18.
- Keating, S. *et al.* (2000) *Prog. Biomed. Optics (SPIE)* **3913**:128-137.
- Levine, L.M. *et al.* (1997) *Anal. Biochem.* **247**:83-8.
- Li, Z. *et al.* (2000) *J. Biomol. Screen.* **5**:31-7.
- Lynch, B.A. *et al.* (1997) *Anal. Biochem.* **247**:77-82.
- Lynch, B. *et al.* (1999) *Anal. Biochem.* **275**:62-73.
- Owicki, J.C. (2000) *J. Biomol. Screen.* **5**:297-306.
- Parker. G.J. *et al.* (2000) *J. Biomol. Screen.* **5**:77-88.
- Pope, A.J. *et al.* (1999) *Drug Discovery Today* **4**:350-62.
- Sportsman, J.R. *et al.* (1997) *High Throughput Screening: the Discovery of Bioactive Substances*, pp. **389-400**. Marcel Dekker, New York.
- Wu, P. *et al.* (1997) *Anal. Biochem.* **249**:29-36.
- Wu, J.J. *et al.* (2000) *J. Biomol. Screen.* **5**:23-30.
- Zhao, G. *et al.* (1999) *Antimicrob. Agents Chemother.* **43**:1124-8.

Limitations of FP

FP requires relatively large changes in molecular volume for maximum signal (change in mP value). The FP value increases with molecular weight, but reaches a plateau level dependent on the fluorescence lifetime of the fluorophore. For a discussion of the impact of lifetime on FP values, see Pope *et al.* (1999). For example, the lifetime of fluorescein is 4 nanoseconds, which is about the same amount of time required for a small molecule (<10 kDa) to randomize its orientation relative to the incident plane of excitation energy. The resulting fluorescence will therefore be depolarized. Binding of this molecule to a larger one will slow down the tumbling of the complex and the fluorescence will remain polarized. A lifetime of 4 nanoseconds is not optimum for observing the binding of a large protein (>30 kDa) to other proteins because the fluorescence is already highly polarized.

Being a ratiometric technique, FP is resistant to absorbance or color quenching from library compounds. However, fluorescence from these library compounds can cause artifacts. This can be dealt with directly by pre-reading the fluorescence in a well before addition of the fluorescent reagent. The background fluorescence can then be subtracted out before the FP value is calculated. Performing background subtraction on individual wells is often not possible during a primary screen, but usually performed in follow up screening on "flagged" compounds that demonstrated significant changes in fluorescence intensity. The probability of finding background fluorescence problems is reduced at higher wavelengths. Therefore using red-shifted probes in place of fluorescein will minimize background fluorescence interference.

Table 1-2 (adapted from Pope *et al.*, 1999) provides a brief listing of advantages and disadvantages of the most prominent homogeneous fluorescence detection methods.

Table 1-2. Advantages and disadvantages of fluorescent formats for homogeneous high throughput screening assays.		
Method	Advantages	Disadvantages
Fluorescence Intensity (FLINT)	• Simple • Suitable for fluorogenic assays • Readily miniaturized	• No information for quality control • Sensitive to inner-filter and auto-fluorescence interference
Fluorescence Polarization (FP)	• simple, predictive • insensitive to inner-filter effects • ratiometric technique • suitable for small (<10 kDa) ligands	• local motion effects • suitability limited by lifetime of dye, ligand size, and molecular weight change • can suffer from auto-fluorescence
Fluorescence Resonance Energy Transfer (FRET)	• suitable for short inter/intramolecular distances (<5 nm) • range of available donors and acceptors	• requires multiple labels • sensitivity to inner-filter and auto-fluorescence interference • limited to short distances to obtain high signal changes
Time-Resolved Energy Transfer (TR-FRET)	• robust • suitable for long distances (5–10 nm) • reduced autofluorescence interference	• requires multiple complex labels • limited choice of donors/acceptors

• Pope, A.J. *et al.* (1999) *Drug Discovery Today* **4**:350-62.

Receptor-Ligand Binding

Introduction

Fluorescence polarization is ideal for the study of small molecule fluorescent ligands binding to receptors. Due to the significant difference in size of the ligands and receptors, the increase in polarization upon binding is relatively large. The first application in this section describes the binding of a fluorescent ligand to recombinant steroid hormone receptors. In this application, the fluorescent ligand is a high-affinity steroidal compound containing a covalently linked fluorophore. Illustrated in Figure 2-1, displacement of the fluorescent steroid by competitor compounds serves as the basis for high throughput screening assays. The other application involves the interaction between a protein domain and a fluorescein-labeled peptide. Peptide/protein interactions are particularly suitable for FP because the design of the fluorescein-labeled peptide ligands is straightforward (Chapter 3 describes further applications with fluorescently labeled peptides).

Figure 2-1. Displacement of a fluorescently labeled steroid from a receptor by a competitor compound is the basis for a FP-based high throughput screening assays.

Receptor:Ligand Competitor Displaced Ligand Receptor:Competitor

High Polarization Low Polarization
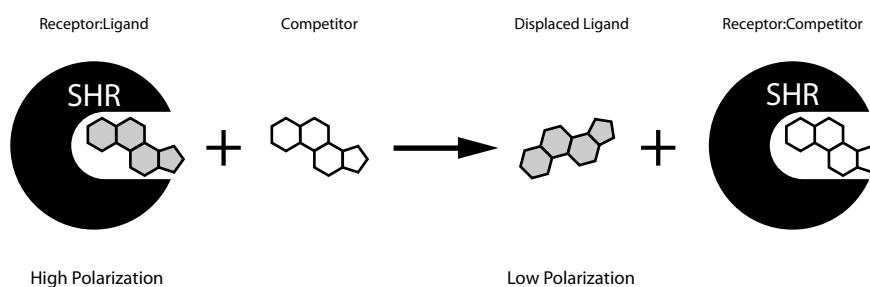
Competitive Binding Assays for Nuclear Receptors

Competition binding experiments are used to analyze binding affinities of nonfluorescent ligands. One can compare the relative binding affinities (IC_{50}) of two or more unlabeled ligands or, when the K_d of the labeled ligand is known, calculate the K_i of the unlabeled ligands. The great benefit of competition experiments is the ability to determine binding affinities without requiring labeling the ligand under study.

• Bolger et al. (1998) Environ. Health Perspect. **106**:551-7.

• Ciocca, D.R. and Roig, L.M. (1995) Endocr. Rev. **16**:35-62.

• Farhat, M.Y. et al. (1996) FASEB J. **10**:615-24.

• Kuiper, G.G. et al. (1996) Proc. Natl. Acad. Sci. USA. **93**:5925-30.

• Turner, R.T. et al. (1990) Endocr. Rev. **15**:275-300.

Analysis of the competition binding curve yields IC_{50} values, which is the concentration of unlabeled ligand necessary to displace 50% of the tracer from the receptor. IC_{50} values are dependent on the experimental system, the concentration of the tracer, and the receptor concentration; therefore, they are not easily compared if these parameters vary between experiments. Obtaining IC_{50} values under identical conditions is an excellent approach if the aim of the study is to compare the relative affinities of a series of ligands. In the typical competition experiment, tracer is incubated with receptor and various concentrations of the unlabeled competing ligand. As the concentration of unlabeled ligand increases, it competes with the tracer for receptor binding. As the fraction of tracer that is bound to the receptor drops, there is a corresponding drop in the polarization value.

Competition experiments performed using FP require relatively high receptor concentrations in order to bind significant amounts of the fluoresceinated ligand. FP competition experiments should be designed such that the [receptor]/K_d ratio is at least 1, so that the starting polarization value will represent at least 50% of the maximal shift. Note that IC_{50} values determined will be greater than the actual K_i values. See Chapter 8 for a detailed discussion of the relationship between IC_{50} and K_i.

In the following example, the affinities of estrogen receptor ligands are assessed by their ability to displace Fluormone™ ES2 from a complex of Fluormone™ ES2 with hERα (Figure 2-2) or hERβ (Figure 2-3). ER/Fluormone™ ES2 complex is added to the compound being tested, which has been diluted in Estrogen Screening Buffer. After a 2 hour incubation, the polarization values are measured. By adding the complex to a serial dilution of the test compound, a competition curve can be generated from which an IC_{50} can be determined.

	IC_{50} (nM)
■ DES	5.4
▲ 4-OH Tamoxifen	29.6
▼ Estradiol	5.7
◆ Tamoxifen	60.9
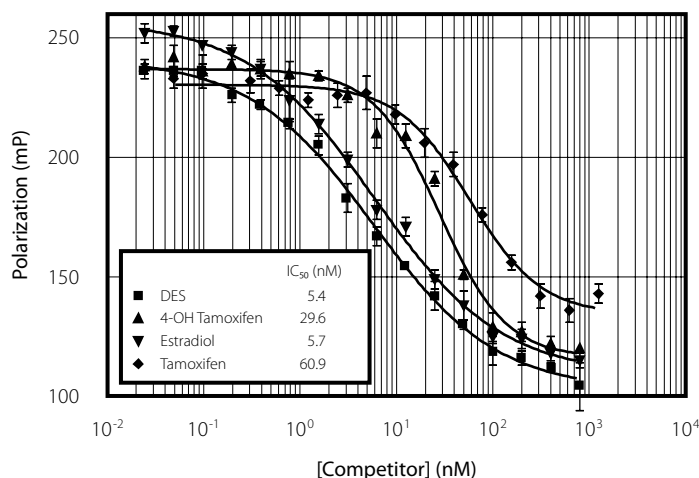
Figure 2-2. Estrogen Competitor α Screening Assay. Serial dilutions of four known competitors for ER were prepared in estrogen screening buffer from 10 mM DMSO stocks. A mixture of ERα and Fluormone™ ES2 was added to the diluted inhibitors to final concentrations of 13 nM ERα and 1 nM Fluormone™ ES2. After a 2-hour incubation at 25°C, fluorescence polarization was measured on a TECAN Polarion using the recommended fluorescein filter set. Binding parameters were calculated from non-linear regression using Prism® from GraphPad Software, Inc.

Figure 2-3. Estrogen Competitor β Screening Assay. Estrogen competitors were assayed as described in Figure 2-2, using ERβ as the receptor component. ERβ and Fluormone™ ES2 were added to the diluted inhibitors to a final concentration of 10 nM ERβ and 1 nM Fluormone™ ES2. Fluorescence polarization was measured as described above. Note that the calculated IC_{50} values are not precisely comparable to those in Figure 2-2, due to slight differences in receptor concentrations.

Competitors	IC_{50} (nM)
■ Tamoxifen	188
▲ DES	3.5
▼ 4-OH Tamoxifen	26.1
◆ Estradiol	5.6
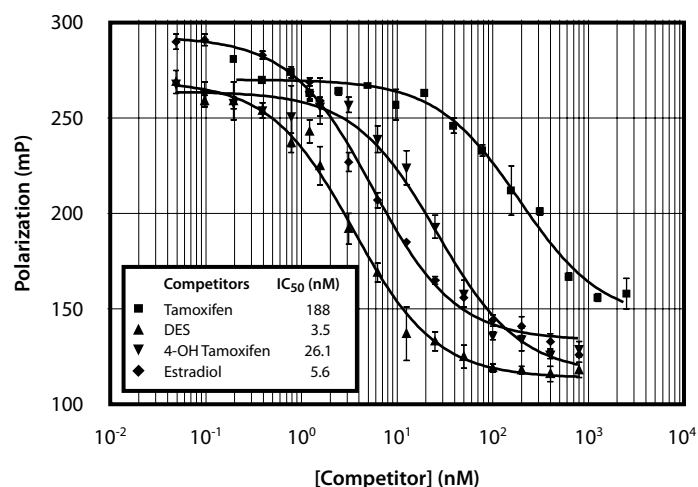
GST-Src Homology Domain Binding to a Fluorescein-Phosphopeptide

A phosphorylated peptide (TSTEPQpYEEIENL) was labeled with fluorescein. The glutathione-S-transferase/Src homology domain fusion (GST-SH2/SH3) was serially diluted in 11 tubes, then the fluorescein-labeled phosphopeptide (F-phosphopeptide) was added to each tube and incubated at room temperature (approximately 25°C) for 18 hours. As a negative control, fluorescein-labeled nonphosphorylated peptide was added to a second serial dilution series of GST-SH2/SH3. Anisotropy values were then measured in all reaction tubes. The data are presented in **Figure 2-7**. The F-phosphopeptide bound the SH2/SH3 domain with high affinity (K_d= 42 nM), while the fluorescein-labeled non-phosphorylated peptide did not bind to the SH2/SH3 domain, as expected. This binding interaction is known to require the phosphotyrosine residue for high-affinity binding (Sudol, 1998).

Figure 2-7. Fluorescein-labeled Peptides Binding to GST-SH2/SH3. A glutathione-S-transferase/Src homology domain fusion was serially diluted from 700 to 0.5 nM in 0.5 mL volumes. Fluorescein-labeled phosphorylated peptide (squares) or fluorescein-labeled unphosphory-lated peptide (triangles) was added to each reaction tube and anisotropy was measured on a Beacon® 2000 after a 2 hour incubation at 25°C. Binding parameters were calculated from non-linear regression using Prism® from GraphPad Software.
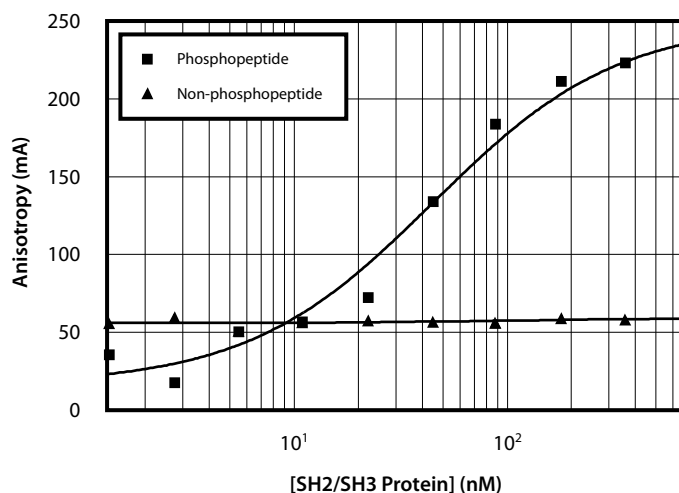
•   Sudol, M. (1998) *Oncogene* 17:1469-74.

CHAPTER 3
Immunoassays

Introduction

Interactions between polyclonal or monoclonal antibodies and their antigens can be studied by exploiting the advantages of FP, leading to assays that rival or exceed ELISAs in sensitivity and ease of use. Homogeneous, competitive fluorescence polarization immunoassays (FPIAs) differ from radioimmunoassays and ELISAs in one important aspect: FPIAs require no separation of the free and bound tracer. FPIAs are similar to other competitive FP assays in that antibody and fluorescent antigen are combined so that the majority of antigen is bound with a high polarization value. As increasing concentrations of unlabeled antigen are added, a greater fraction of the labeled antigen is displaced, and the observed polarization value decreases. The sensitivity of the FPIA primarily depends on the affinity of the antigen/antibody pair, the instrument sensitivity, and the difference in polarization value between the free and bound forms of the labeled antigen. As with other FP applications, FPIAs are best suited for the quantitation of antigens smaller than 10 kDa. This is fortuitous because ELISAs usually require multiple antigenic sites and are often ill-suited for the detection of small molecules such as drugs and peptides.

Most immunoassays require at least two steps: formation of an immunocomplex followed by physical separation of bound from free antigen. Both radioimmunoassays and ELISAs involve one or more steps in which the liquid phase is removed and the retained immunocomplex is extensively washed to remove unbound and non-specifically bound molecules. During each wash, bound antigen can be released from the solid phase. Uncontrolled leaching of bound material causes both systematic and sample-to-sample variability. The amount of loss depends on several variables, such as the type of solid matrix used, the equilibrium constants of the immunocomplexes, and the duration, temperature, and solvent conditions of the wash. Not all of these factors can be tightly controlled. FPIAs, on the other hand, require no separation step, totally avoiding this problem.

Detection of Kinase Activity using
Fluorescence Polarization (Patent Pending)

The phosphorylation of proteins by kinase cascades (and subsequent dephosphorylation by phosphatases) is critical to the normal regulation of biological mechanisms. Kinases catalyze the transfer of a high-energy phosphoryl group from ATP to the hydroxyl group of a serine, threonine, or tyrosine residue. Phosphorylation appears to be the "master" biochemical reaction in signal transduction, and it has been estimated that at least one-third of the proteins in the average mammalian cell are phosphorylated (Hunter, 1995). Traditional *in vitro* kinase assays are often tedious and involve either large amounts of ^{32}P or labor-intensive (and sometimes ambiguous) western blotting. Neither of these techniques allows for the real-time observation of the phosphorylation event. Invitrogen is a leader in the development of simple, sensitive, non-radioactive, non-immobilized, protein kinase assays designed for basic research and inexpensive high throuput screens of kinase activity modulators.

Tyrosine Kinase Assays

The regulated and reversible phosphorylation of tyrosines is critical to the normal regulation of many biological mechanisms, including cell growth, proliferation, differentiation, motility, transcription, synaptic function, and metabolism (Hunter, 1995; Hunter, 1998; Pawson, 1995). There have been more than 95 PTKs and 55 tyrosine phosphatase genes found in humans (Hunter, 1998). Defects in these signal transduction pathways can result in a number of human diseases, including cancer (Hunter, 1995; Pawson, 1995). For example, in the case of the epidermal growth factor (EGF) receptor, over-expression and mutation have been associated with some of the most incurable cancers, including glial and pancreatic tumors (von Deimling *et al.*, 1995; Friess *et al.*, 1996; Wong *et al.*, 1992). Unfortunately, the development of anticancer agents based on the inhibition of EGF binding to the receptor have had limited success (Eppstein *et al.*, 1989). Efforts to screen for new tyrosine kinase inhibitors had been hindered by the lack of robust, high-throughput tyrosine kinase assays. With Invitrogen's kinase kits, these screens are simpler, cheaper, and accelerate the development of new drug candidates.

Traditional tyrosine kinase assay formats include ELISA- and RIA-based methods. These methods require immobilization of reaction components on plates, and multiple separation and washing steps, making them difficult to implement in HTS. Although radioactive methods are very sensitive, they create significant radioactive waste. Newer homogeneous methods now in use include SPA (Scintillation Proximity Assay; Baum *et al.*, 1996) and HTRF® (Homogeneous Time-Resolved Fluorescence; Kolb *et al.*, 1998). SPA is a radioactive technique requiring immobilization and HTRF® requires labeling and characterizing multiple assay components.

FP-based tyrosine kinase Assays are homogeneous, fluorescent assays requiring no immobilization, making them ideal for HTS. In the FP-based kinase assay, a fluorescently-labeled phosphopeptide (tracer) and phosphopeptides generated during a kinase reaction compete for binding to anti-phosphotyrosine antibodies (anti-pY Ab). When there are no kinase reaction products present, the tracer will be bound by the anti-pY Ab, resulting in a high FP value. However, after a tyrosine kinase reaction has occurred, reaction products displace the tracer from the anti-pY Ab, resulting in a decreased FP value. Thus, the reduction in FP value is directly related to the amount of the tyrosine kinase activity.

- Baum, E.Z. *et al.* (1996) *Anal. Biochem.* **237**:129-34.
- Eppstein, D.A. *et al.* (1989) J**.** *Cell. Physiol.* **141**:420-30.
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- Hunter, T. (1995) *Cell* **80**:225-36.
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- Kolb, A.J. *et al.* (1998) *Drug Discovery Today* **3**:333-42.
- Pawson, T. (1995) *Nature* **373**:573-80.
- von Deimling, A., Louis, D.N., Wiestler, O.D. (1995) *Glia* **15**:328-38.
- Wong, A.J. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* **89**:2965-9.

Measurements can be made in real time, allowing the observed enzymatic activity to be monitored both kinetically or in an endpoint assay format. This description is illustrated in **Figure 3-1**:

Figure 3-1. In an FP-based kinase assay, the tracer and phosphopeptides generated during a kinase reaction compete for binding to anti-pY Ab. When there are no kinase reaction products present, the tracer will be bound by the anti-pY Ab, resulting in a high FP value. However, after a tyrosine kinase reaction has occurred, reaction products displace the tracer from the anti-pY Ab, resulting in a decreased FP value. The reduction in FP value is directly related to the amount of the tyrosine kinase activity.
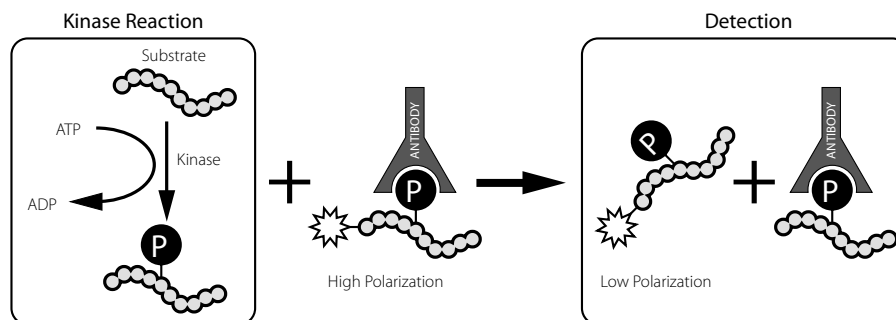
In **Figure 3-2**, the change in FP values due to tyrosine kinase activity was assayed using members of the src family, including Src, Src N1, Lyn A, and Lyn B. The complete reaction/detection system was run in a final volume of 50 μL under the following final reaction conditions: 20 mM HEPES (pH 7.4), 2 mM $MgCl_2$, 5 mM $MnCl_2$, 50 μM Na_3VO_4, 2 ng/mL poly [Glu, Tyr] 4:1. Enzyme was titrated down from 100 ng/mL using two-fold dilutions. ATP was added to a final concentration of 10 μM to start the reaction and after 90 minutes, the reaction was stopped by the addition of 5 mM EDTA. Detection components were added and the signal was measured on a TECAN Ultra. Percent phosphorylation was determined by calculating the difference in polarization values between unbound tracer (all of the antibody is bound to the unlabeled phosphotyrosine generated by the kinase reaction and the tracer is free in solution; low polarization) and bound tracer (all of the F-phosphopeptide tracer is bound by the anti-phosphotyrosine antibody; high polarization).

A kinase assay based on FP can be used to detect autophosphorylation of the kinase, as well as the phosphorylation of any additional substrates that are present. In the case of the EGF receptor, autophosphorylation provides a significant amount of the competitor phosphotyrosines. In this situation, measuring the K_m for the peptide substrate is still possible by running an assay with no substrate and measuring the shift in mP that occurs due to autophosphorylation of the enzyme.
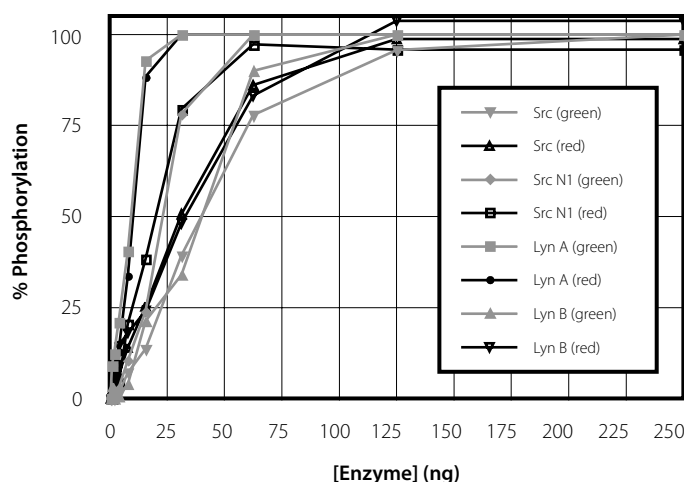
Figure 3-2. Titration of Four Src Enzymes in the PTK Green and PTK Red Kits. The complete reaction/detection system was run in a final volume of 50 µL under the following final reaction conditions: 20 mM HEPES (pH 7.4), 2 mM MgCl$_2$, 5 mM MnCl$_2$, 50 µM Na$_3$VO$_4$, 2 ng/mL poly [Glu, Tyr] 4:1, and enzyme titrated down from 100 ng/mL in two-fold dilutions. 10 µM final ATP was added to start the reaction, and after 90 minutes the reaction was stopped by the addition of 5 mM EDTA. Detection components were added and the signal was measured on a TECAN Ultra.

Serine/Threonine Kinase Assays

The phosphorylation of serine and threonine residues in proteins by serine/threonine kinases is central to the normal regulation of many biological mechanisms including the modulation of membrane structure, receptor desensitization, transcriptional control, cell growth and differentiation, and the mediation of immune responses like inflammation. Serine/threonine kinases such as protein kinase C (PKC) family members also play a role in memory and learning, as well as in various pathological processes (Nishizuka, 1986; Nishizuka, 1992; Dekker and Parker, 1994). A number of studies have suggested that inappropriate activation of PKCs can contribute to cancer, inflammation, viral infection, immune and CNS disorders, cardiovascular malfunction, vascular complications of diabetes, and insulin resistance (Hu, 1996). Therefore, identifying inhibitors to serine/threonine kinases, such as the PKC family members, is essential to developing new therapies for these diseases.

Like the FP-based tyrosine kinase assay, the principle behind the serine/threonine kinase assays is competition. Fluorescein-labeled phosphopeptide tracers and any unlabeled phosphopeptide products generated during a serine/threonine kinase reaction will compete with each other for binding to anti-phosphoserine or anti-phosphothreonine peptide-specific antibodies. In a reaction mixture containing no phosphopeptide products, a significant portion of the fluorescent tracer will be bound by the antibody, resulting in a high polarization value. However, in a reaction mixture containing phosphopeptide products, some of the tracer will be displaced from the antibody and the emission signal will become depolarized. Therefore, the change in polarization is directly related to the amount of serine/threonine kinase activity in a reaction.

Figure 3-3 is a representative example of a competition curve from one of Invitrogen's serine/threonine kinase assays, the IκB-α pSer 32 Assay Kit (Invitrogen Part No. P2827 and P2828). At high concentrations of phosphopeptide, the polarization shifts from 250 mP to 45 mP. This change in polarization occurs because the F-phosphopeptide tracer that is initially bound by anti-phosphoserine peptide specific antibody (high polarization) is displaced by the phosphopeptide competitor. However, the concentration of competitor phosphopeptides is lowered by dilution in subsequent wells and less antibody is bound to the unlabeled phosphopeptide competitor. Therefore, the F-phosphopeptide tracer remains bound to the anti-phospho-serine peptide-specific antibody. If this antibody is left out of the reaction, then the F-phosphopeptide: anti-phosphoserine peptide-specific antibody complex cannot form (low polarization = 45 mP). If the antibody and F-phosphopeptide tracer are mixed in the absence of competitor phosphopeptide, then the complex can form (high polarization = 250 mP). These data demonstrate the specificity of the antibody by showing that neither the non-phosphopeptide competitor or the non-specific phosphopeptide competi-

• Dekker, L.V., Parker, P.J. (1994) *Trends Biochem. Sci.* **19**:73-7.

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tor (p36) can displace the F-phosphopeptide from the anti-phosphoserine peptide-specific antibody at concentrations relevant to a kinase reaction.

As shown in **Figure 3-4,** Invitrogen's Protein Kinase C Assay Kit (Invitrogen Part No. P2747 and P2748)

Figure 3-3. IκB-α Assay Kit Competition Curves. Different IκB-α–derived peptides (14 amino acids each) compete with the fluorescein-labeled phosphoserine 32 IκB-α tracer for binding sites on the anti-phosphoserine 32 IκB-α antibody. Competitors were serially diluted, and the antibody and tracer were added to each well. Differential displacement of the tracer by the three different IκB-α peptides peptides is shown.
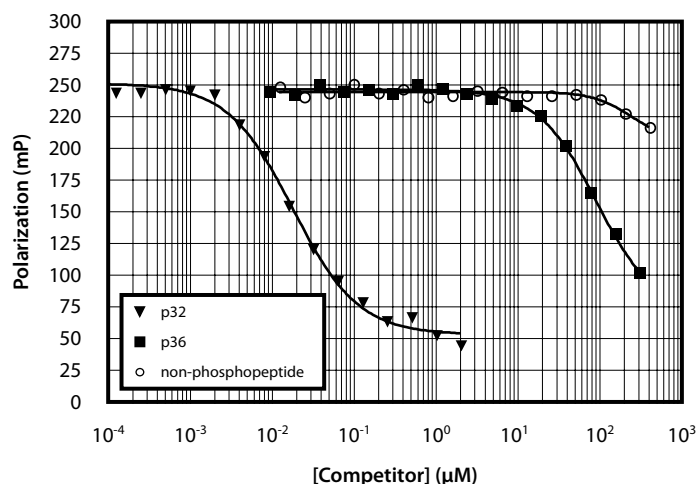
can be used to determine IC_{50} values for inhibitors of specific serine/threonine kinases such as staurosporine, a potent inhibitor of PKC isoforms (Tamaoki *et al.*, 1986). Briefly, in a round-bottom, black 96-well plate, PKC isoforms (15 ng PKCα, 300 ng PKCβI, 300 ng PKCβII, 17 ng PKCγ, 117-ng PKCδ, or 65 ng PKCε) were individually incubated with serial dilutions of staurosporine (Calbiochem; San Diego, CA) for 5-minutes at room temperature. ATP was then added to start the reactions. Final reaction conditions (for 50 µL total reaction volume) were: 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 100 µM $CaCl_2$, 0.1 mg/mL phosphatidylserine, 0.02 mg/mL diacylglycerol, 2 µM peptide substrate, 2X PKC fluorescent phospho-peptide tracer, 50 µM sodium vanadate, 0.02% NP40, and 10 µM ATP. After a 90-minute incubation at room temperature, 50 µL of a quench/detection mixture (containing 130 mM EDTA and the anti-phosphoserine antibody) was added to stop each reaction and initiate the competition for antibody binding. Results were measured on a TECAN Polarion Instrument and non-linear regression analysis was performed on a semi-log plot of the data. The IC_{50} values of staurosporine were shown to be less than 10 nM for all PKC isoforms tested.

• Tamaoki, T. *et al.* (1986) *Biochem. Biophys. Res. Commun.* **135**:397-402.
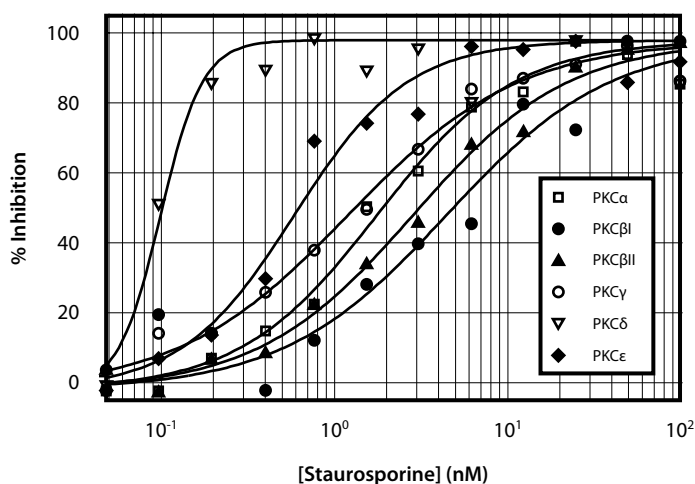
Figure 3-4. In a round-bottom, black 96-well plate, PKC isoforms (15 ng PKCα, 300 ng PKCβI, 300 ng PKCβII, 17 ng PKCγ, 117 ng PKCδ, or 65 ng PKCε) were individually incubated with serial dilutions of the inhibitor staurosporine (Calbiochem; San Diego, CA) for 5 minutes at room temperature. ATP was then added to start the reactions. Final reaction conditions (for 50 μL total reaction volume) were: 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 100 μM $CaCl_2$, 0.1 mg/mL phosphatidylserine, 0.02 mg/mL diacylglycerol, 2 μM peptide substrate, 2X PKC fluorescent phosphopeptide tracer, 50 μM sodium vanadate, 0.02% NP40, and 10 μM ATP. After a 90-minute incubation at room temperature, 50 μL of a quench/detection mixture (containing 130 mM EDTA and the anti-phosphoserine antibody) was added to stop each reaction and initiate the competition for antibody binding. Results were measured on a TECAN Polarion Instrument and non-linear regression analysis was performed on a semi-log plot of the data. The IC_{50} values of staurosporine were shown to be less than 10 nM for all PKC isoforms tested.

Detection of Tyrosine Phosphatase Activity Using Fluorescence Polarization (Patent Pending)

Dephosphorylation of proteins is an important regulatory mechanism of many biological processes. The enzymes that catalyze these reactions, protein phosphatases, also wreak havoc with basic research and development on protein kinase-regulated events. Contamination of cell extracts, immunoprecipitates, and column fractions with active phosphatases can seriously impair the progress of a research project. The use of protein phosphatase inhibitors can be critical to the success of molecular and cellular biology research. Therefore, determining if a sample is free of phosphatase activity and the development of new and more powerful phosphatase inhibitors are important areas in research and development, quality control, and the search for new therapeutics.

The general principles described for kinase detection can also be applied to a phosphatase assay which, like the kinase assay, can be monitored as either a kinetic or end-point assay. Phosphatase assays are slightly different from the competition-based PTK assays described earlier in that reaction progress is monitored using direct binding rather than competition. Tyrosine phosphatases cleave the phosphate from phosphotyrosine, so as the tracer is dephosphorylated by a phosphatase, it is no longer recognized by the anti-pY Ab, increasing the free fraction and decreasing the polarization of the sample.

An FP-based protein phosphatase assay offers a simple, rapid, very sensitive, and non-radioactive means for detecting phosphatase contamination in QC applications or studying the role of phosphatases in basic research. This assay can also be used to screen for novel phosphatase inhibitors. The principle of this assay is dependent on the equilibrium binding of fluorescein-labeled phosphopeptides (F-phosphopeptides) by anti-phosphotyrosine antibodies (anti-pY Ab). When there are no active phosphatases present in a sample, a significant portion of the F-phosphopeptide tracer will be bound by anti-phosphotyrosine antibodies and the equilibrium is undisturbed, resulting in a high polarization. However, when a phosphatase reaction has occurred, the F-phosphopeptide will be dephosphorylated by the enzyme, which alters the equilibrium binding, and the anti-phosphotyrosine antibodies will be unable to bind to the resultant F-peptide. This increases the amount of free tracer and decreases the polarization of the sample. Thus, the reduction in polarization is directly related to presence and

amount of phosphatase activity. Measurements can be made in real-time, allowing the researcher to follow the activity of the enzyme kinetically. This reaction is illustrated in **Figure 3-5**:

Figure 3-5. When there are no active phosphatases present in a sample, a significant portion of the F-phospho-peptide tracer will be bound by anti-phosphotyrosine antibodies and the equilibrium is undisturbed, resulting in a high polarization. However, when a phosphatase reaction has occurred, the F-phosphopeptide will be dephosphorylated by the enzyme, which alters the equilibrium binding, and the anti-phosphoty-rosine antibodies will be unable to bind to the resultant F-peptide. This increases the amount of free tracer and decreases the polarization of the sample.

High Polarization   Low Polarization
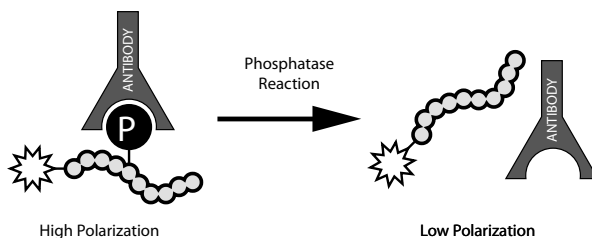
Protein Tyrosine Phosphatase Assay

In **Figure 3-6,** the enzymatic activity of T-Cell Protein Tyrosine Phosphatase (TC PTP) was measured in a dose-dependent manner by incubating different concentrations of TC PTP (New England Biolabs; Beverly, MA), from 0.05 U/µL to 0.0005 U/µL, with 50 µL of a mixture of anti-phosphotyrosine antibodies and fluorescein-labeled phosphopeptides. The final reaction volume was 100 µL. Two control assays were performed: one did not receive TC PTP, while the other was supplemented with 50 µM Na_3VO_4 (Sigma, St. Louis, MO), a potent phosphatase inhibitor. Increasing concentrations of TC PTP result in an increased rate of dephosphorylation, which is indicated by a decrease in polarization. This change in polarization was dependent on the presence of TC PTP and could be completely inhibited by 50 µM Na_3VO_4.

Figure 3-6. The enzymatic activity of T-cell protein tyrosine phosphatase (TC PTP) was measured in a dose-dependent manner by incubating different concentrations of TC PTP, which was from New England Biolabs (Beverly, MA). The TC PTP concentration ranged from 0.05 U/µL to 0.0005 U/µL. Each well also contained 50 µL of a mixture of anti-phosphotyrosine antibodies and fluorescein-labeled phospho-peptides in a final volume of 100 µL. Polarization values were measured continuously on a Beacon® 2000 instrument after the addition of TC PTP (kinetic mode). Two control assays were performed: one did not receive TC PTP, while the other was supplemented with 50 µM of the potent phosphatase inhibitor Na_3VO_4. In both cases, no dephos-phorylation occurred and the polar-ization remained high throughout the experiment.

- ● No TC PTP
- □ 0.05 U/µL TC PTP + 50 µM Na_3VO_4
- ◆ 0.0005 U/µL TC PTP
- ▼ 0.0025 U/µL TC PTP
- ▲ 0.005 U/µL TC PTP
- ■ 0.05 U/µL TC PTP
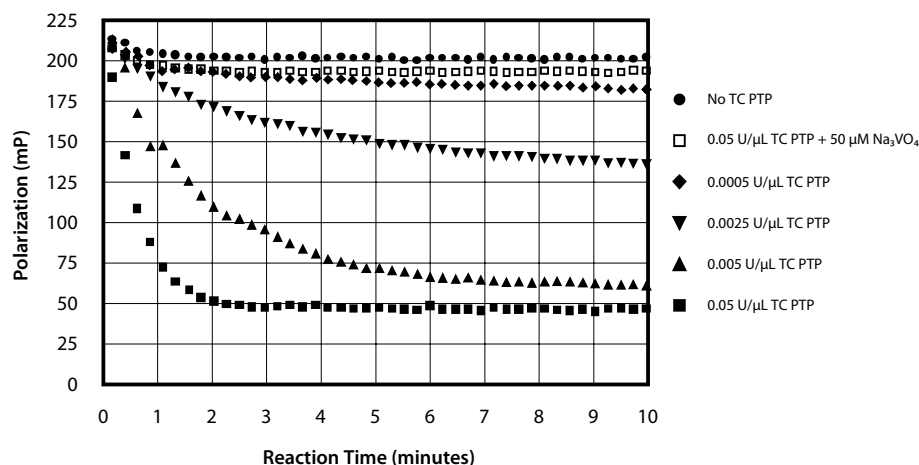
Inhibition of TC PTP by Sodium Vanadate

To demonstrate the usefulness of an FP-based phosphatase assay in screening for phosphatase inhibitors, the IC_{50} value for Na_3VO_4 and TC PTP was determined in **Figure 3-7**. In a black, 96-well plate (DYNEX; Chantilly, VA), Na_3VO_4 (Sigma; St. Louis, MO) was serially diluted 2-fold into 24 wells from a starting concentration of 500 nM in a volume of 50 µL. TC PTP was then added to each well so that the final concentration of the enzyme would be 0.005 U/µL per assay. To start the reaction, 50 µL of a mixture of anti-phosphotyrosine antibodies and fluorescein-labeled phosphopeptides was added, and each reaction was incubated for 15 minutes. At the end of the incubation, 10 µL of 550 µM Na_3VO_4 was added to quench all of the reactions, and the polarization for each sample was measured on a TECAN Polarion. Non-linear regression analysis of a semi-log plot was used to analyze these data. The IC_{50} of the ubiquitous phosphatase inhibitor Na_3VO_4 was determined to be 4 nM for TC PTP.
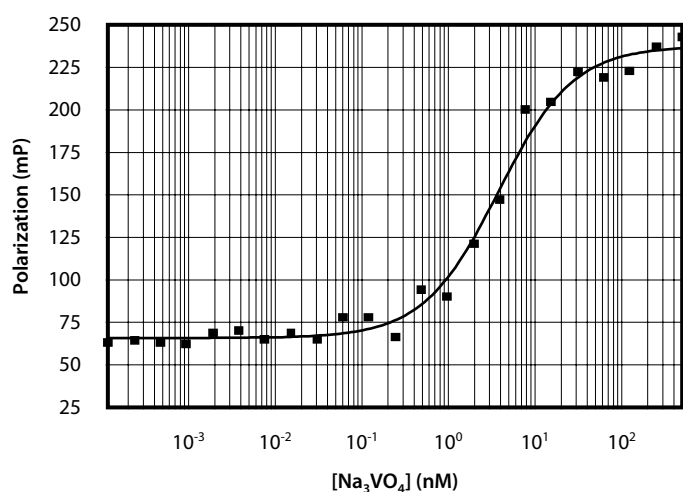
Figure 3-7. The IC_{50} value for Na_3VO_4 and TC PTP was determined in the following manner: In a 96-well plate, Na_3VO_4 was serially diluted 2-fold into 24 wells from a starting concentration of 1000 nM in a volume of 50 µL. TC PTP (New England Biolabs; Beverly, MA) was then added to each well so that the final concentration of the enzyme would be 0.005 U/µL per assay. To start the reaction, 50 µL of a mixture of anti-phosphotyrosine antibodies and fluorescein-labeled phosphopeptides was added, diluting the Na_3VO_4 concentration 2-fold. Each reaction was incubated for 15 minutes. At the end of the incubation, 10 µL of 550 µM Na_3VO_4 was added to quench all of the reactions, and the polarization for each sample was measured on a TECAN Polarion. Non-linear regression analysis of a semi-log plot of the data was used to determine an IC_{50} of approximately 4 nM.

Quantitation of Antigen: Fluorescence Polarization Immunoassay for Epidermal Growth Factor

Introduction

In this application, we developed a simple FPIA for Epidermal Growth Factor (EGF), a 53-amino acid polypeptide hormone that regulates cell proliferation and may facilitate wound healing.

Materials and Methods

In duplicate, a 1:10 dilution of mouse EGF polyclonal rabbit antiserum (Sigma Chemical; St. Louis, MO) was serially diluted in PBS-BSA (50 mM potassium phosphate (pH 7.4), 150 mM NaCl and 0.1 mg/mL BSA) and added to 12 x 75 mm borosilicate tubes (Invitrogen Part No. 2182). One set of tubes also received 1 nM mouse EGF (Molecular Probes; Eugene, OR). The final volume in all tubes was 1 mL. The background fluorescence of each tube was measured using the Beacon® Analyzer. Fluorescein-labeled EGF (F-EGF; Molecular Probes, Eugene, OR) diluted in PBS-BSA was then added to each tube at a final concentration of 100 pM. The tubes were mixed gently and allowed to incubate at room temperature for 60 minutes. The fluorescence polarization of each tube was again determined and the blank-corrected polarization values were plotted versus the amount of antibody in the absence and presence of 1 nM unlabeled EGF.

Results and Discussion

As shown in **Figure 3-8**, 0.4 µL of a 1:10 dilution of anti-EGF antibody gave the greatest difference in polarization value (*i.e.,* resulted in the maximum shift upon the addition of unlabeled EGF) between the two binding curves. This amount of antibody was chosen for construction of the standard curve. Note that the antibody concentration giving the maximal response range did not correspond to the antibody concentration that resulted in the maximum polarization value for the F-EGF in the *absence* of the unlabeled molecule.

Figure 3-8. Antibody Optimization for EGF FPIA. Increasing amounts of anti-EGF antibody were added to tubes containing 100 pM fluorescein-EGF in the absence (open circles) and the presence (closed circles) of 1 nM unlabeled EGF.
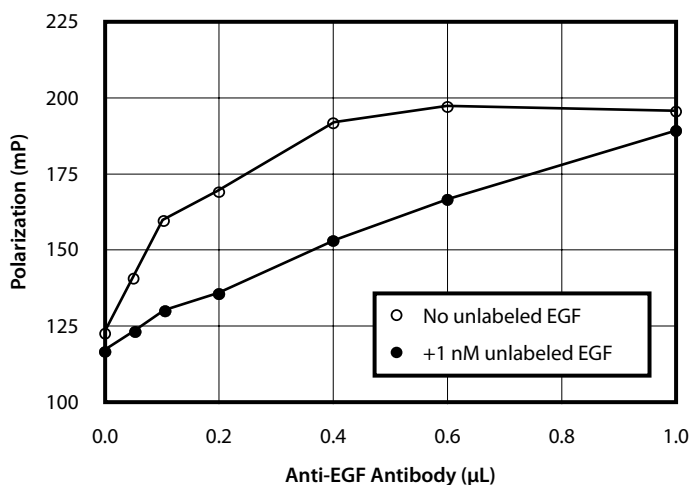
To develop the EGF standard curve, a range of EGF concentrations was added to duplicate tubes containing 0.4 µL of a 1:10 dilution of EGF Ab in a total volume of 1 mL of PBS-BSA. The tubes were gently mixed, and fluorescence background of each tube was determined using the Beacon® Analyzer. F-EGF, diluted in PBS-BSA, was added to each tube to a final concentration of 100 pM. The tubes were gently mixed and allowed to incubate at room temperature for 60 minutes. Background corrected fluorescence polarization values were determined for each tube using the Beacon® Analyzer. Averaged results are shown in **Figure 3-9**.
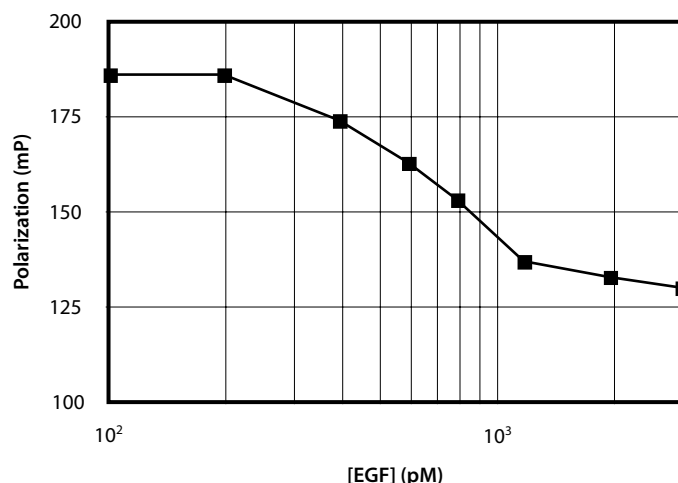
Figure 3-9. Standard Curve for EGF FPIA. 100 pM fluorescein-EGF and 0.4 µL of a 1:10 dilution of anti-EGF antibody was added to tubes containing a serial dilution of EGF. The polarization values were read on a Beacon® Analyzer.

Development of the FPIA involved two steps:

1. Determination of the amount of antibody that results in the maximal response range (**Figure 3-8**).

2. Construction of a standard curve (**Figure 3-9**).

The sensitivity of FPIAs is affected by many factors including the sensitivity of the instrument used, the affinity of the labeled antigen for the antibody, the fluorescence intensity of the tracer, and the background fluorescence of the sample. Because sensitivity is also a function of the dynamic range (*i.e.,* the difference in polarization between the free antigen and antibody:antigen complex), FPIAs are best suited for the quantitation of smaller antigens. Standard ELISAs are often difficult to develop for small molecules because of the requirement for multiple antigenic sites.

As with any competition binding assay, the tracer need not be identical to the unlabeled antigen, nor do the affinities of the tracer:antibody and unlabeled antigen:antibody interactions need to be identical. The affinity of the tracer:antibody interaction is the most important determinant of assay sensitivity. It is often the limiting factor in FPIA development. Care should be taken when choosing the tracer molecule. The lower the affinity, the more unlabeled antigen required to produce a response (*i.e.,* displacement of the tracer from the complex), which lowers the sensitivity of the assay.

The study of antibody-antigen interactions using FP is not limited to quantitative assays. For example, FP can be used to characterize the binding of monoclonal antibodies and their antigens, which is especially useful during the screening of monoclonal libraries.

Useful Literature

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Characterization of a Single-chain Antibody: Binding of Fluorescein to Single-chain Anti-fluorescein

Introduction

This application describes the binding characterization of a single-chain antibody directed against fluorescein. The fluorescein binds directly to the antibody, and it is thereby severely quenched: the intensity of free fluorescein is greater than the intensity of the bound fluorescein. This system is ideal for a discussion of the data transformation necessary when fluorescence intensity of the free and bound states are significantly different.

Materials and Methods

• Single-chain anti-fluorescein antibody (F_{sc} Ab)

• 100 nM fluorescein in bovine gamma globulin (BGG)/Phosphate Buffer

• Beacon® BGG/Phosphate Buffer (Invitrogen Part No. P2013)

Using 18 tubes, single-chain anti-fluorescein antibody (F_{sc} Ab) was serially diluted (from 3 µM to 23 pM) using BGG/Phosphate Buffer (Invitrogen Part No. P2013) in a total volume of 1.2 mL. Five microliters of 100 nM fluorescein in bovine gamma globulin (BGG)/Phosphate Buffer were added to each tube, for a final concentration of 500 pM. Tubes were incubated for 30 minutes at room temperature (equilibrium was previously determined to be reached at 10 minutes). Anisotropy of each tube was measured using the Beacon® Analyzer.

We have used two different methods for the correction of polarization and anisotropy, when the intensities of the bound and free tracer are not equal (*i.e.*, $Q_f \neq Q_b$). Both methods are described.

Method A (from Dandliker *et al.*, 1981)

The bound to free ratio of the fluorescent tracer can be defined as:

$$\textbf{Equation 3.1:} \qquad \frac{F_b}{F_f} = \left[\frac{A - A_f}{A_b - A} \right] \times \frac{Q_f}{Q_b} = \frac{M - A_f}{A_b - M}$$

Where:

F_b = molar concentration of bound fluorescent tracer

F_f = molar concentration of free fluorescent tracer

Q_b = molar fluorescence of bound tracer

Q_f = molar fluorescence of free tracer

A = measured anisotropy

A_b = anisotropy of bound tracer

A_f = anisotropy of free tracer

M = the corrected anisotropy

• Dandliker, W.B. *et al.* (1981) *Meth. Enzym.* **74**:3-28.

Solving for M:

Equation 3.2: $\quad M = \dfrac{\{[(A - A_f)/(A_b - A)](Q_f/Q_b)(A_b)\} + A_f}{1 + [(A - A_f)/(A_b - A)(Q_f/Q_b)]}$

A, A_f and Q_f are easily obtained as direct measurements. A_b and Q_b are the anisotropy and intensity of the completely bound tracer, respectively. Because we cannot achieve complete binding, these values must be estimated as the asymptote of the respective binding isotherm (*i.e.*, A versus receptor concentration or Q versus receptor concentration). Any standard binding data analysis software can easily make these calculations.

Previously, we have suggested using two linear transformation equations to determine the limit polarization and intensity rather then estimating these values as the asymptotes of the binding isotherms. We have included those equations for those who wish to use them. In the example below, we have used anisotropy instead of polarization.

In **Equation 3.3**, Q_b is determined by plotting the values of Q (the observed intensity) vs. $(Q_f - Q)$/(receptor concentration). The y-intercept is Q_b (see **Figure 3-10**).

Equation 3.3: $\quad Q = Q_b + \dfrac{Q_f - Q}{\textbf{Receptor Concentration}}$

Figure 3-10. Determination of Q_b. Q_b is the y-intercept. Only values corresponding to the eight highest antibody concentrations were plotted. Q_b was determined to be 3102.
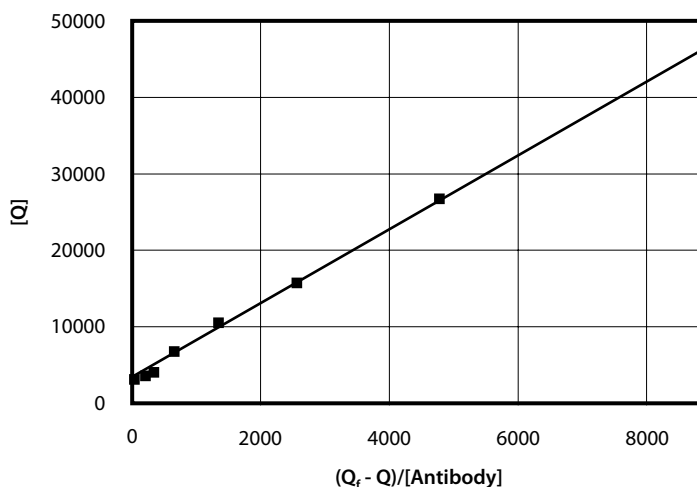
Equation 3.4 was used to solve for A_b by plotting A (the observed polarization values) versus $[(Q_f)(A-A_f)]/[(Q_b)$(receptor concentration)$]$. The y-intercept of this plot is A_b (see **Figure-3-11**).

Equation 3.4: $\quad A = A_b - \left[\dfrac{(Q_f)(A - A_f)}{(Q_b)(\textbf{Receptor Concentration})}\right]$
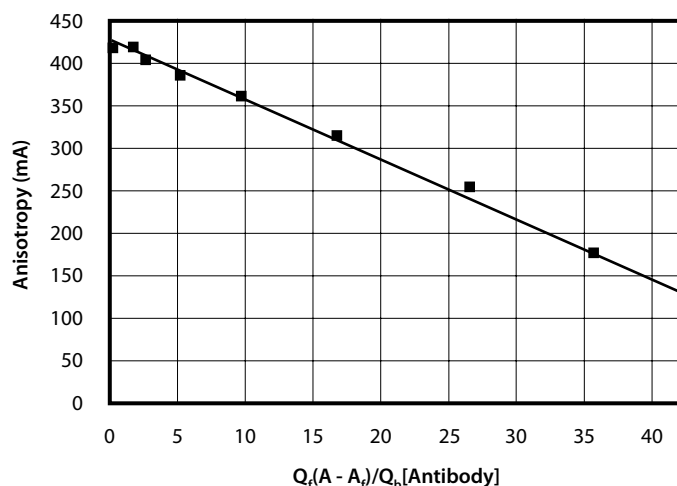
Method B (from Lundblad *et al.*, 1996)

When $Q_f \neq Q_b$, the corrected fraction of bound tracer (F_b) is defined as:

Equation 3.5: $$\text{Corrected } F_b = \frac{A - A_f}{(A_b - A)(Q_b/Q_f) + A - A_f}$$

Where:

   A = the observed anisotropy

   A_f = the anisotropy of the ligand in the free state

   A_b = the anisotropy of the ligand in the bound state

   Q_b = the intensity of the ligand in the bound state

   Q_f = the intensity of the ligand in the free state

To solve **Equation 3.5**, Q_f, A_f, Q_b, and A_b need to be determined. As described in **Method A**, Q_f and A_f are measurements of the free tracer in the absence of its binding partner, and Q_b and A_b can be estimated from the semi-log binding curves or through the linear transformations obtained from **Equations 3.3** and **3.4**.

Results and Discussion

Intensity, antibody concentrations, observed millianisotropy values, and corrected millianisotropy values are shown in **Table 3-1**. Note that as the fraction of bound fluorescein increases, the intensity drops.

Both the observed and corrected sets of values were plotted, and the isotherms were fit using a non-linear least squares curving fitting program (**Figure 3-12**). The K_d for the fluorescein:anti-fluorescein antibody interaction calculated from the raw data was 165 nM. After the data were corrected for changes in intensity, the calculated K_d was 11 nM, a difference of 15-fold. The raw data (triangles) were adjusted for intensity changes by **Method B** to yield the corrected curve (squares).

• Lundblad, J.R., Laurance, M. and Goodman, R.H. (1996) *Mol. Endo.* **10**:607-12.

Table 3-1. Intensity, antibody concentrations, observed millianisotropy values and corrected millianisotropy values.

Q	[mAb] (nM)	mA	Corrected mA
80614	0.029	17.5	29.3
76955	0.057	17.5	30.1
78318	0.114	18.0	40.6
77680	0.229	18.4	51.4
75802	0.458	19.0	64.4
70144	0.916	19.5	72.6
76880	1.831	20.0	83.0
60098	3.662	22.5	124.5
45861	7.324	26.8	176.3
26545	14.6	40.8	270.0
15362	29.3	70.5	344.1
10386	58.6	112.8	381.9
6710	117.2	177.7	404.8
3885	234.4	255.7	416.9
3485	468.8	315.4	422.1
3154	937.5	362.5	425.0
3191	1875	387.0	426.3
2481	3750	405.0	427.1
1984	15000	420.0	427.7
1974	30000	394.3	426.6

Figure 3-12. Equilibrium Binding Isotherms of Fluorescein to Anti-fluorescein Antibody.
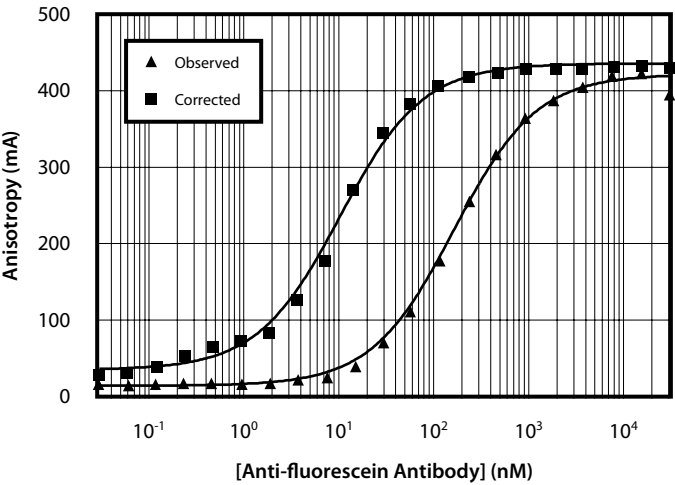
CHAPTER 4
DNA-Protein Binding

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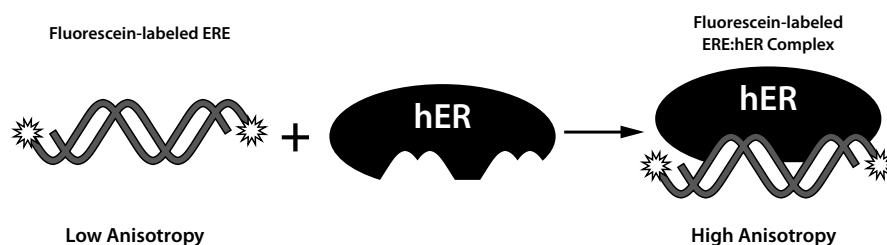
Figure 4-1. Principle of the F-ERE: hER binding assay. When measuring the polarization of ERE-F in the absence of hER, the labeled DNA is free to rotate in solution (low polarization value). When the ERE-F is bound by hER, the large complex is slow to rotate in solution (high polarization value).

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.
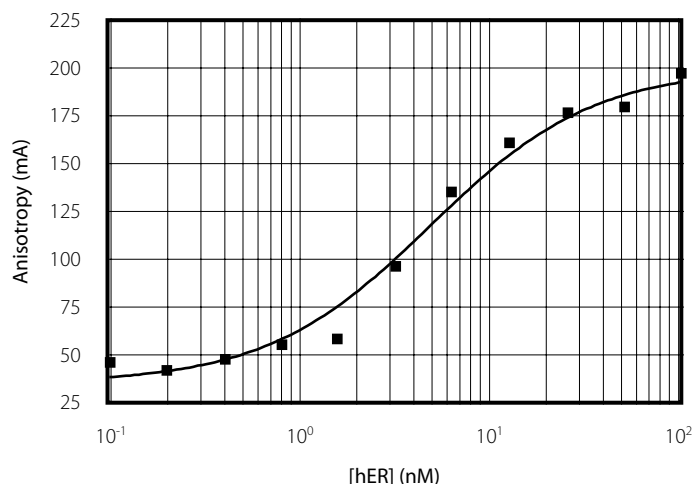
Binding of *trp* Repressor to a Fluorescein-labeled *trp* Repressor Operator (U.S. Patent No. 5,445,935)

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
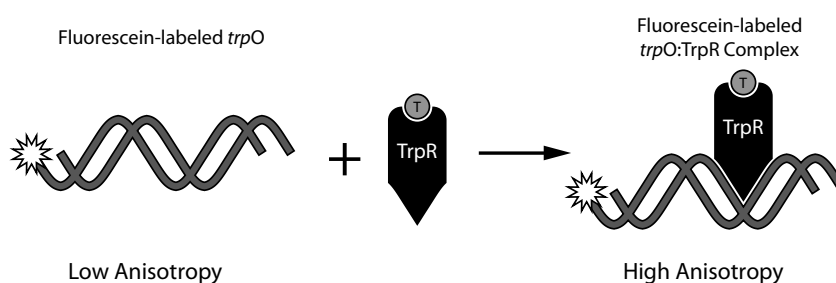
The oligonucleotides used for this experiment are shown below. The Trp25-F oligo was labeled with the fluorescein attached to its 5' end via a 6-carbon spacer:

```
Trp25-F:   5'-F-ATCGAACTAGTTAACTAGTACGCAA-3'
Trp25-A:      3'-TAGCTTGATCAATTGATCATGCGTT-5'
```

The 25 base-pair, double-stranded oligonucleotide (*trp*O-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 over 30 minutes. The TrpR

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

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

was serially diluted from 1 µM to 3.9 pM in 1.0 mL of 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 TrpR 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.
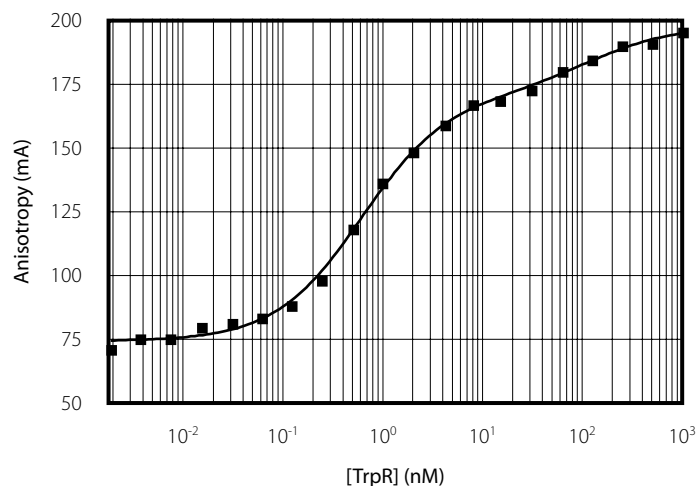
Figure 4-4. Trp Repressor Binding to *trp*O-F. Recombinant Trp repressor, purified from *E. coli*, was serially diluted from 1 µM to 3.9 pM in 10 x 75 mm tubes containing 10 mM potassium phosphate, 0.1 mM EDTA (pH 7.6), 4 mM tryptophan, 10% glycerol. *trp*O-F was added to each tube to a concentration 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 D14 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.
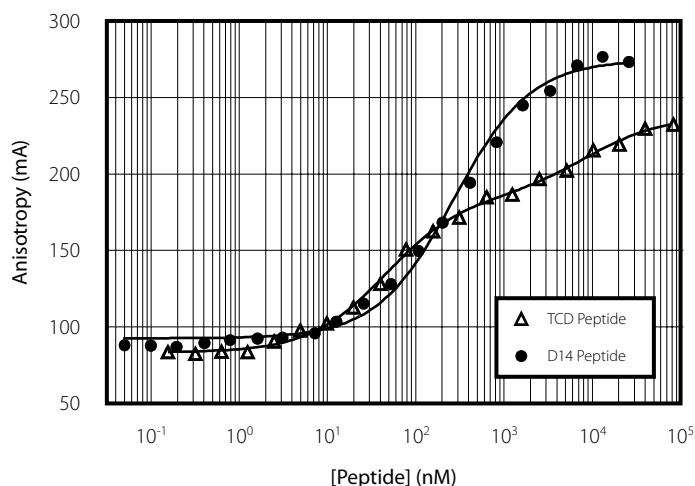
These data are in close agreement with the work of Brasier and Kumar (1994) who estimated the K_d for the D^{14} peptide-DNA binding as 283 nM and the K_d for TCD peptide-DNA binding as 36 nM using a gel mobility shift assay. Analysis of the gel-shift data did not identify the TCD low-affinity binding 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.
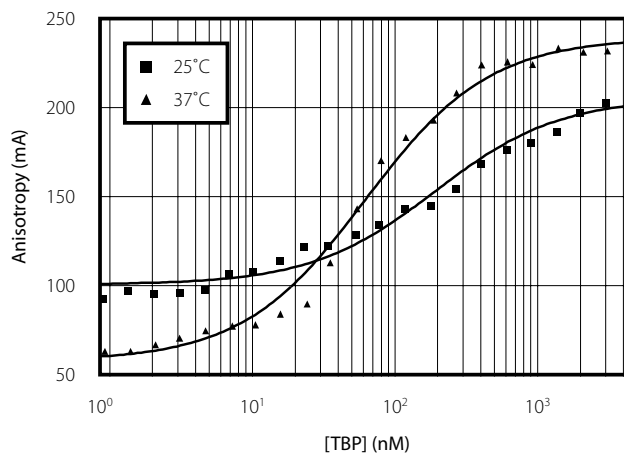
Acknowledgment

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 MW of 35,000 Da and binds to the DNA with a K_d of 2.5 nMp

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.

CHAPTER 5
Degradative Assays

Degradative Assays

The least appreciated types of FP assays are the degradative assays. In these assays, a large fluorescent substrate is acted upon by an enzyme that cleaves the substrate into smaller fragments. The substrate is large and therefore has a high polarization value while the fragments necessarily have lower polarization values.

In classic FP receptor:ligand binding assays, the labeled tracer has only two polarization states: free or bound. The observed polarization value is a weighted average of these two extremes. In degradative assays, this is not the case. Generally, unless the enzyme has a very specific cleavage activity, the large substrate will be cleaved into a complicated mixture of small fluorescent and non-fluorescent fragments. The curve of polarization versus enzyme concentration represents the weighted average of possibly dozens of fluorescent fragments.

The key advantage of FP degradative assays is the same as for binding assays: there is no need to separate the degraded from undegraded substrate. For instance, in the classic protease assay using fluoresceinated casein as the substrate, the enzyme activity is determined by separating the undigested substrate, which can be precipitated with trichloroacetic acid (TCA), from the smaller casein fragments, which cannot be precipitated with TCA. This precipitation step is unnecessary in FP assays. Another advantage of the FP assay is that enzyme activity can be followed in real-time with the proper instrumentation.

While proteases are the most widely considered FP can follow, in theory, many other degradative enzymes. In this chapter, we will show examples of degradative FP assays with proteases, DNases, RNases, and amylases, but other types of enzymes can be formatted for FP assays as well.

Degradative FP assays can also be designed with very small substrates. In our original paper on the design of a fluorescent casein FP protease assay (Bolger and Checovich, 1994), we postulated about using biotin and fluorescein at opposite ends of the cleavage site on a small peptide. Upon completion of the assay, avidin is added to the reaction mixture, raising the molecular weight of the intact substrate (the avidin will also bind to the cleaved biotin labeled fragment, but this is invisible to FP). The observed polarization value will be a weighted average of the intact fluorescein-peptide-biotin-avidin substrate and the fluorescein-peptide cleaved fragment. Subsequently, Levine and colleagues put this suggestion to practice (Levine *et al.*, 1997).

• Bolger, R.E. and Checovich, W.C. (1994) *BioTechniques* **17**:585-9.

• Levine *et al.* (1997) *Anal. Biochem.* **247**:83-88.

A Quantitative Amylase Assay in Malt and Molasses Samples

Introduction

Proteases, amylases, and lipases each play important roles in the processing of dairy products, starches and sugars, fruits and vegetables, baking products, and malt used for brewing. Enzymes are used for proper processing (*e.g.*, α-amylase activity on starch from malt), are responsible for contaminating activity in additives (*e.g.*, endogenous protease activity in spice additives), and contribute to flavor formation (*e.g.*, lipase additives in cheese processing).

The ability to follow enzyme activities during food processing can potentially reduce costs by allowing for early rejection of unsuitable raw ingredients, improvements in process optimization, and more rigorous, consistent, and accurate quality control of final products. Any new assays should meet several different criteria, including adequate sensitivity, ease of use, speed, and statistical robustness. The ideal assay set would also share a common technology in order to simplify training of laboratory personnel.

Amylases and related starch-degrading enzymes, like proteases, play a role in a myriad of food processes, including starch conversion, filtration, fermentable sugar production, juice clarification, and liquefaction. Amylase is especially important to the malt and brewing industries. Amylase concentrations in malt play a significant role in product taste and texture, and only malts with a certain amylase activity are used in beer-brewing. The amylase activity FP-based assay described in this section is based on an FP assay originally designed to detect amylase activity in clinical serum samples (Hofman and Shaffar, 1985). It was developed to measure high amylase activities in malt samples and low, contaminating levels of amylase activity in molasses. In the first case, the presence of amylase activity in malt is essential to the brewing process; in the second, contaminating amylase activity in molasses leads to changes in texture over time. While not detailed here, other enzymes (*e.g.*, lipases, pectinases, cellulases, etc.) which degrade larger substrates to small fragments are also candidates for FP assays.

Materials and Methods

Amylose (Sigma Chemical Co.) was labeled with FITC according to the method of De Belder and Granath (1973). Malt barley was a kind gift from Dr. Cynthia Henson of the Cereal Crops Research Unit, USDA, and the Department of Agronomy, University of Wisconsin-Madison.

Malt samples were prepared as follows: 5 grams of malt barley were ground in a coffee grinder for one minute. One gram of ground malt was added to 20 mL Tris-buffered saline (pH 7.4). The sample was gently mixed for one hour at room temperature and then 1.0 mL of the extract was spun at $10,000 \times g$ for 5 minutes in a microcentrifuge. The supernatant was transferred to polystyrene tubes.

The basis of the fluorescence polarization amylase assay described below is identical to that of the other enzyme assays described in this chapter. A large, fluorescently-labeled amylase substrate (F-amylose) was incubated with varying concentrations of purified α-amylase in PBS (pH 7.4) at room temperature for 10 minutes to overnight. Changes in polarization values were determined using a Beacon® 2000 Fluorescence Polarization System. For the comparison of malt extracts, F-amylose was incubated with 2 μL of a 1:10 dilution of each malt extract in PBS for 50 minutes at room temperature. For analysis of molasses, pure molasses was diluted 1:10 or 1:100 with PBS and incubated with F-amylose for up to 24 hours. Color in the molasses samples was accounted for by background subtraction and did not interfere with the assay.

Figure 5-1. Protease Assay Theory. In the absence of protease, the labeled protein substrate emits light that is emitted is highly polarized (due to the size of the casein molecule). As the substrate molecules are hydrolyzed to smaller, faster rotating, labeled peptides, the emitted light is less polarized and the fluorescence polarization value decreases.

Results and Discussion

The amylase activity standard curve is shown in **Figure 5-9.** Amylase activity was linear over 2–3 logs. We also followed the kinetics of amylase activity and found that breakdown of the fluorescein-labeled amylose substrate was minimal over 24 hours (data not shown).

We also examined amylase activities of three molasses samples, and compared them to a positive control spiked with an excess of amylase, and a negative control (**Table 5-3**). Differences in color attributed to the molasses were accounted for in the analysis. The lower polarization value for the amylose substrate when it is completely broken down was 70 mP, while 268 mP was the upper limit for totally intact substrate. Changes in polarization values correlated well with amylase activity in the molasses.

Our FP assay measured amylase activity at both very high levels (in malt extracts) and very low levels (in molasses samples). Relative amylase activities in malt extracts determined by FP were comparable to those activities determined by the malt industry standard method. For instance, malts with high 20 DU activities had the greatest changes in polarization. We allowed the reactions to proceed for 60 minutes, but shorter periods would have been sufficient because amylase activity was so high in the samples that were tested. While we reported our amylase activities in terms of ΔmP, a standard curve, using malt extracts with qualified 20 DU activities, could be used to convert millipolarization values to 20 DU numbers.

Analysis of molasses by FP differed from the malt analysis, because amylase in malt represents a low level contamination, not the primary enzymatic activity. It was therefore necessary to incubate the reactions for a longer period (*i.e.*, overnight). Color of molasses samples did not prove to be a problem at 1:100 dilution although the diluted samples were dark. Polarization is relatively insensitive to slight color, and colored samples can be accounted for in the blanking process. The assay, using the Beacon® 2000 Fluorescence Polarization System, correctly predicted the amylase activity in the samples relative to their previously determined amylase activity.

One of the most appealing advantages of FP is that the same instrumentation and assay design can be used in a variety of situations. For instance, with starch degrading enzymes, only the fluorescent substrate need be changed in order to assay different activities. It is conceivable that judicious choice of substrate could lead to the development of unique assays for β-amylase, debranching enzyme, and even α-glucosidase.

Acknowledgments

We thank Dr. Cynthia Henson (Cereal Crops Research Unit, USDA and the Department of Agronomy, University of Wisconsin-Madison) and Dr. Donald Carpenter (Kraft Foods, Glenview, Illinois) for their thoughtful advice and discussions.

• Hofman, M. and Shaffar, M. (1985) *Clin. Chem.* **31**:1478-80.

• De Belder, A.N. and Granath, K. (1973) *Carbohydr. Res.* **30**:375-8.
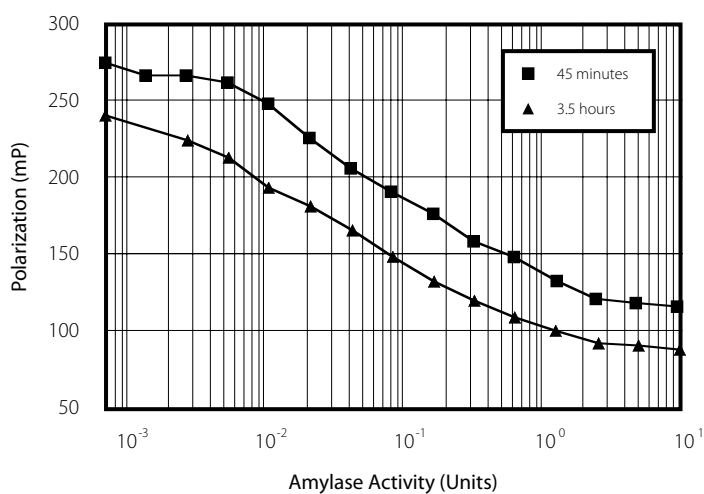
Figure 5-9. Amylase Activity Standard Curve. To assess the appropriateness of this assay for the characterization of malts, we compared the activity of ten common malts. Table 5-2 compares amylase activity in a variety of different malts by two methods: the change in polarization values of the F-amylose substrate and malt industry standard 20 Dextrinizing Units (20 DU) α-amylase activities. The latter were determined previously by the USDA, ARS Cereal Crops Research Unit, Madison, WI using a standard method (20 DU method, α-amylase activity, American Society for Brewing Chemist Standard Method, 1976 Edition). We did not construct a standard curve and so the comparison of activities determined by the two methods must be relative.

Table 5-2. Comparison of Amylase Activity in Malt Extracts. Reactions done at room temperature for 60 minutes.		
Malt Barley	Change in polarization (mP)	α-amylase activity (20 DU)
M 81	66	37.8
ND 13300	63	39.8
Stander	60	36.7
Morex	58	34.2
M 75	57	36.1
Excel	55	34.8
Barbless	40	24.5
ND 14161	41	28.7
Y3250-6	39	28.6
SM 90514	27	21.4

Table 5-3. Amylase Activity in Molasses Samples.	
Sample	Change in Polarization (mP)
Negative control (zero point)	268
Positive control (very high activity)	70
Sample I (>60 Units/kg)	158
Sample IV (20 Units/kg)	230
Sample V (<60 Units/kg)	199

CHAPTER 6
DNA Hybridization and Detection

Fluorescence Polarization Detection of DNA Hybridization

Introduction

Nucleic acid hybridization is widely used to characterize and quantitate RNA and DNA sequences. It is often desirable to determine that complementary oligonucleotides properly anneal or to optimize hybridization conditions for oligonucleotides used in the PCR. Fluorescence polarization provides a direct, simple, and non-radioactive method of measuring the extent of nucleic acid hybridization without filter immobilization.

This application demonstrates simple and specific nucleic acid hybridization using fluorescence polarization. A fluorescein-labeled oligonucleotide is shown hybridizing specifically to its complementary strand, but not to an unrelated DNA sequence. The oligonucleotides are heat-denatured and allowed to cool while fluorescence polarization values are measured continuously. The basic elements of this assay are illustrated in **Figure 6-1**:

Figure 6-1. Nucleic acid hybridization using fluorescence polarization. A fluorescein-labeled oligonucleotide hybridizes specifically to its complementary strand, but not to an unrelated DNA sequence. The oligonucleotides are heat-denatured and allowed to cool while fluorescence polarization values are measured continuously.
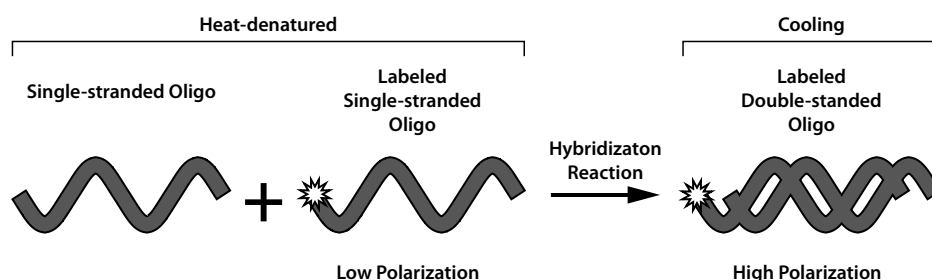
Materials and Methods

Three oligonucleotide primers were prepared: A 5' fluorescein-labeled T7 primer (T7-fl, 5'–Fl-TAATACGACTCACTATAGGG–3'), an unlabeled Sp6 primer (Sp6, 5'–ATTAAGGTGACACTATAGAA–3'), and an unlabeled complementary T7 primer (T7-comp, 5'–CCCTATAGTGAGTCGTATTA–3'). The fluorescein-labeled primer was synthesized using standard phosphoramidite chemistry with a six-carbon spacer to the fluorescein and was purified by HPLC. Each lyophilized primer was resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and the absorbance at 260 nm was used to determine the DNA concentration. Then, 10 pmol (60 ng) T7-fl DNA was added to 2.0 mL phosphate buffered saline (PBS) for a final concentration of 5 nM. The 2.0 mL sample was heated at 85°C in a 12 × 75 mm borosilicate test tube (Invitrogen Part No. P2182) for 10 minutes. While the tube was heating, a kinetic program was started on the Beacon® System. PBS buffer at room temperature was used to blank the Beacon® Analyzer, and the instrument was set to measure polarization values continuously at 13 second intervals. After heating, the sample tube was wiped free of any external moisture and placed immediately in the Beacon® Analyzer. Polarization values were collected while the solution cooled over a 20 minute period to 37°C. The fluorescent oligonucleotide was also heated in the presence of a complementary oligonucleotide (T7-comp primer) in one tube and a non-complementary oligonucleotide (Sp6 primer) in another. After heating to 85°C, these tubes were each allowed to cool to 37°C in the Beacon® Analyzer while measuring polarization values every 13 seconds.

Results and Discussion

The resultant kinetic data in **Figure 6-2** show that the polarization value of the single-stranded T7-fl oligonucleotide solution rises as the temperature decreases (Δ20 mP within 2 minutes). This increase in polarization is seen in both the T7-fl solution and in the T7-fl/Sp6 solution where non-complementary oligonucleotides are mixed. Theses changes in polarization are due to the changes in the temperature and viscosity of the solutions, and not due to hybridization.
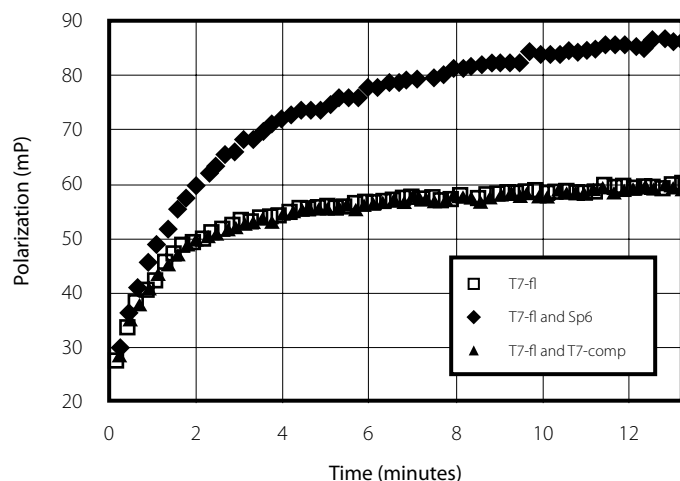
Figure 6-2 Fluorescence Polarization Measurement of DNA Hybridization. Curves for single-stranded T7-fl alone (open squares); T7-fl and Sp6 single-stranded oligonucleotides together (closed triangles); T7-fl and T7-complementary oligonucleotides together (closed diamonds).

When complementary oligonucleotides (T7-fl and T7-comp) are heated and cooled together, the increase in polarization values (86 mP versus 60 mP, final polarization value) was greater than what would be expected from changes in temperature and viscosity alone (*i.e.*, T7-fl alone or T7-fl/Sp6 mixture). This additional increase in polarization is due to hybridization of the complementary strands, which slows the molecular rotation of the labeled oligonucleotide, raising the fluorescence polarization value of the duplex.

Many fluorescence polarization applications involve measuring the increase in size of a small, labeled tracer by virtue of binding to a larger molecule. These results illustrate that fluorescence polarization can be used even if the unlabeled molecule is the same size as the labeled molecule. In this case, both the labeled and unlabeled hybridizing strands were 20 base pairs long. Though the lower size limit for detection of hybridization has not been determined in this experiment, it is reasonable to assume that even smaller oligonucleotides will also work.

Useful Literature

• Murakami, A. *et al.* (1991) *Nuc. Acids Res.* **19**:4097-102.

• Nordlund, T.M. *et al.* (1989) *Biochemistry* **28**:9095-103.

• Murakami, A. *et al.* (1992) *Nuc. Acids Symp-Ser.* **27**:123-4.

Note: Detection of nucleic acid sequences using fluorescence polarization is patented by Zeneca Limited and licensed to Invitrogen Corporation. European Patent No. EP-382,433; other patents pending.

Detection of Amplified DNA by Fluorescence Polarization

Introduction

A fluorescence polarization (FP) based method has been developed for the homogeneous detection of amplified nucleic acid sequences in solution. This detection method is based on the observation that the FP value of a fluorescently-labeled oligonucleotide increases significantly when it hybridizes to its complementary strand. After amplification, a sample of the double-stranded DNA product is denatured by heat and base in the presence of a fluorescein-labeled oligonucleotide (F-oligo). Two specific, 40-base, unlabeled, single-stranded oligonucleotides which hybridize to the target DNA sequences adjacent to the fluorescent probe target sequence are added in excess. These two oligonucleotides are referred to as 'blocking oligos' as their function is to block the two amplified DNA strands from re-annealing and competing with the hybridization of the fluorescent probe. The mixture is cooled and neutralized to allow the F-oligo to hybridize to the target DNA, giving a quantitative measurement of the amount of amplified DNA present. The FP detection method requires no separation or immobilization and can be performed in a matter of minutes. The data presented here demonstrate the speed and sensitivity achieved by the FP-based hybridization assay.

Materials and Methods

To demonstrate the sensitivity of DNA detection using fluorescence polarization, hybridization was measured between a fluorescein-labeled, single-stranded oligonucleotide and increasing amounts of a complementary single-stranded oligonucleotide. It was anticipated that the polarization of the labeled oligonucleotide would increase in proportion to the amount of complementary target present. The F-oligo binding to a complementary 40-mer oligonucleotide is shown in **Figure 6-3**.

Figure 6-3 Fluorescence Polarization Detection of Hybridization Between F-Oligo and a 40-mer Complementary Oligonucleotide. This simple model system using a fluorescein-labeled, 25-base probe and a complementary 40-base oligonucleotide was used to compare the theoretical and practical limits of DNA detection. The complementary oligonucleotide was serially diluted from 10 nM to 156 pM in 7 borosilicate tubes containing 1 ml of 4X SSC buffer at 37°C. The F-oligo was added to each tube to a final concentration of 1 nM. The samples were incubated at 37°C for 2 hours and then the fluorescence polarization was measured on a Beacon Analyzer at 37°C. Polarization values were plotted as a function of complementary DNA concentration.
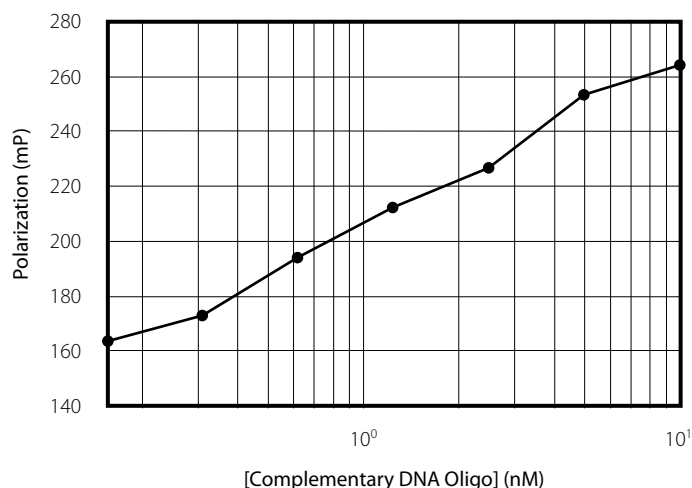
Results and Discussion

To demonstrate that this technique works with a double-stranded target, a single-stranded fluorescein-labeled oligonucleotide was hybridized to double-stranded amplified DNA (**Figure 6-4**).
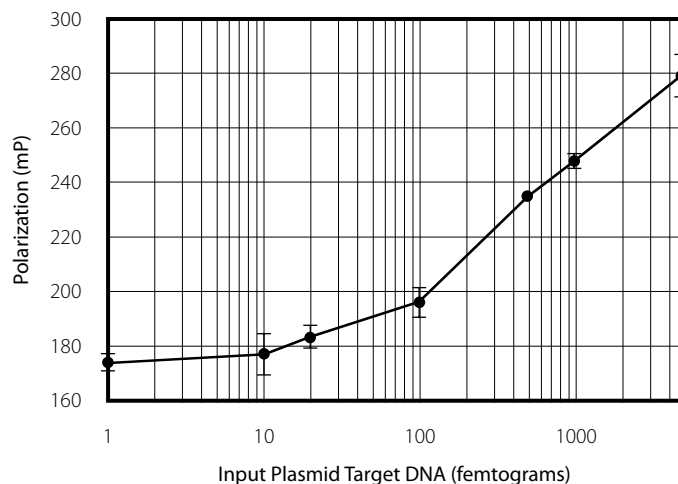
Figure 6-4. Direct Detection of Hybridization Between F-Oligo and PCR-amplified DNA. The target plasmid DNA was serially diluted from 5 picograms to 1.2 femtograms and each dilution was amplified by PCR in a 100 µL reaction. 80 µL of each amplification reaction were added to 10 µL of 1 N NaOH and incubated at 75°C for 30 minutes to denature the DNA. The 90 µL reactions were then added to 1 mL of 50 mM Tris-HCl, 6X SSC, pH 8.0, at 37°C containing F-oligo and blocking oligos. Hybridizations were incubated at 37°C for 30 minutes and then the FP values for each reaction were measured at 37°C on the Beacon® System. The data represent the mean of triplicate experiments, ± 1 standard deviation.

DNA was detected directly from individual PCR reactions. The amplification was performed on a recombinant plasmid in order to detect a low number of plasmid copies in a product derived from the plasmid. One strand of the amplified DNA competes with the fluorescent probe for hybridization to the complementary strand. Two blocking oligonucleotides were included in the hybridization reaction to prevent the two strands of amplified DNA from re-annealing in the regions adjacent to where the probe hybridizes. The polarization value of the F-oligo is proportional to the concentration of the target DNA in a PCR reaction. This technique also works with a 96-well fluorescence polarization format, as shown in **Figure 6-5**.
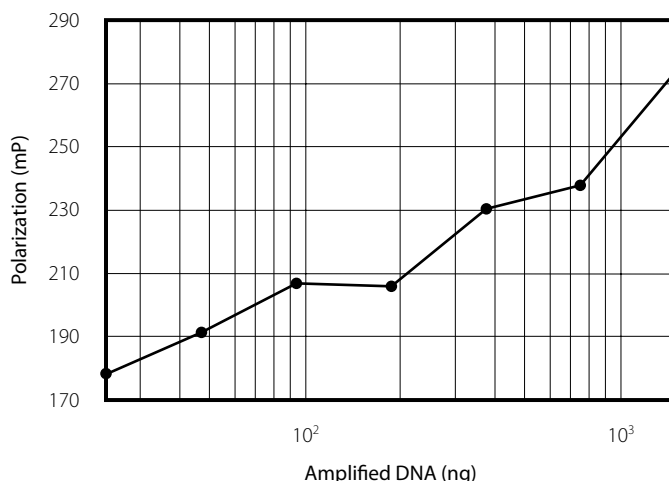
Figure 6-5. Detection of Hybridization Between F-Oligo and PCR-amplified DNA using a 96-well Format. The 239 base-pair amplified DNA was serially diluted in triplicate 90 µL volumes from 1500 to 23 ng on a 96-well microtiter plate. 10 µL of 1 N NaOH were added to each well and incubated at 75°C for 30 minutes to denature the DNA. The F-oligo and blockers were added to each sample in 100 µL of 1 M Tris-HCl, 1 M NaCl (pH 7.5). Hybridization reactions were incubated at room temperature for 10 minutes. FP values were measured on a Fluorolite FPM-2, 96-well fluorescence polarization instrument (Jolly Consulting and Research, Grays Lake, IL).

The experiments presented in this section demonstrate that fluorescence polarization is a rapid, quantitative, and homogeneous method for the detection of amplified DNA. Amplification and detection

take less than 4 hours with very little "hands-on" time required. This technique can easily be used to quantitatively monitor PCR amplification, or can be used as an endpoint detection system. Fluorescence polarization detection of amplified DNA can be used in both single-tube and 96-well formats.

A Quantitative Reverse Transcriptase Assay Using Fluorescence Polarization (U.S. Patent No. 6,100,039)

Introduction

Reverse transcriptases play a key role in the propagation of retroviruses and are therefore key targets for the development of new therapeutics. This assay quantitatively measures the RNA-dependent DNA polymerase activity of reverse transcriptases using a homogeneous, non-radioactive fluorescent assay. An oligo-dT primer is hybridized to a 300 base poly-rA and extended with reverse transcriptase. The RNA:DNA duplex is treated with sodium hydroxide to hydrolyze the RNA strand, leaving the single-stranded DNA. Fluorescently labeled oligo-dA (F-oligo-dA) is then hybridized to the single-stranded DNA, causing a shift in polarization for the fluorescent oligonucleotide. When free, the fluorescent oligonucleotide has a low polarization value but when hybridized to the complementary DNA strand, it has a high polarization value. The shift in polarization is proportional to the amount of reverse transcriptase activity present. The general reaction scheme is diagrammed in **Figure 6-6**:

Figure 6-6. Principle of FP-based RT Assay. This technique quantitatively measures the RNA-dependent DNA polymerase activity of RT using FP. An oligo-dT primer is hybridized to poly-rA and extended with RT. The RNA:DNA duplex is treated with NaOH to hydrolyze the poly-rA template. F-oligo-dA (low polarization value) is hybridized to the single-stranded poly-dT, increasing the FP value.
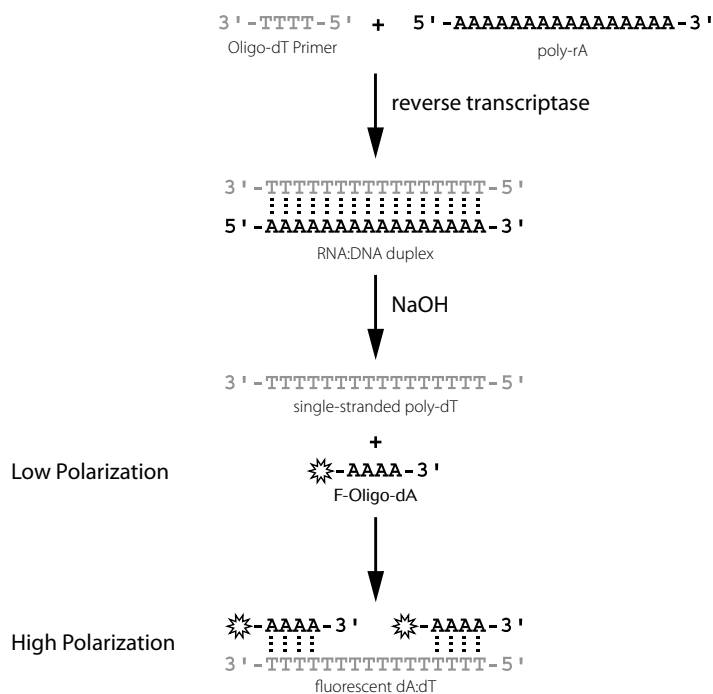
Materials and Methods

The reverse transcriptase activity detection assays were performed on the Beacon® 2000 FP System. The following reagents were purchased from commercial sources: fluorescein-labeled oligo dA (F-oligo-dA; BioSynthesis Corp., Lewisville, TX), AMV Reverse Transcriptase (Promega Corp., Madison, WI), and polyadenylic acid (poly-rA; Pharmacia Biotech, Milwaukee, WI). The assay requires a reverse transcriptase (RT) reaction step followed by an FP-based detection step. The reverse transcriptase reactions were completed using the directions accompanying the enzyme. In the reaction, 20 ng of the Oligo-dT primer was annealed to 1 µg of poly-rA at 70°C for 5 minutes. The annealed reactions were added to an RT mix containing RT buffer and dTTP nucleotides with varying units of reverse transcriptase (30, 15, 7.5, 3.8, and 1.9 Units/reaction). Reactions were incubated at 37°C in a water bath. 5 µL aliquots were quenched at 5, 10, 15, 20, 25, 30, 45, and 60 minutes by adding the aliquots to a tube containing 20 µL of 125 mM NaOH. For the detection step, a 75 µL aliquot of F-oligo-dA in 0.5 M Tris (pH 7.5), was added to each quenched reaction. The samples were incubated for 10 minutes at room temperature.

Results and Discussion

Fluorescence polarization in each sample was measured on the Beacon® 2000 Fluorescence Polarization Instrument. **Figure 6-7** demonstrates the increase in polarization value as polymerase activity continues over time. In addition, the polarization value increases with increasing amounts of enzyme (if time is held constant).
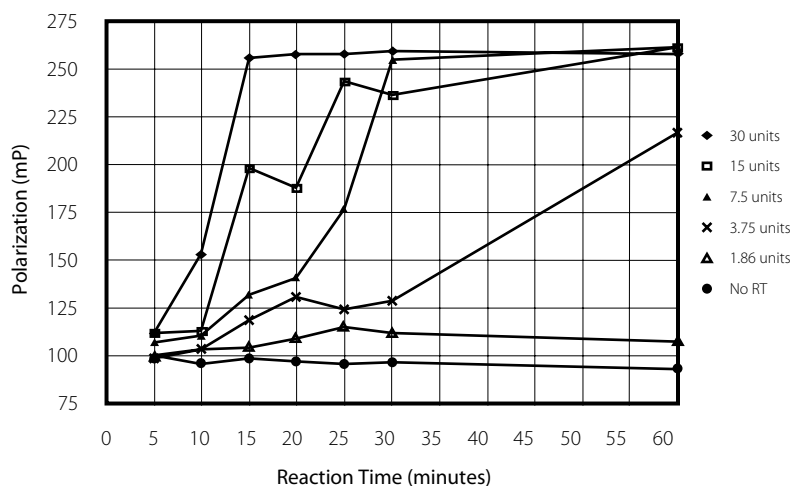
Figure 6-7 Reverse Transcriptase Activity Assay. A fluorescein-labeled oligo-dT (low polarization value) is hybridized to nascent poly-dA, synthesized by reverse transcriptase (RT). Hybridization of F-oligo-dT results in an increase in fluorescence polarization. As more RT is added to the reaction, the rate at which poly-dA is synthesized is increased.

CHAPTER 7

Theory of Binding Data Analysis

Clark's Theory

The quantitation of physical constants defining classical ligand-receptor interaction is dependent on the following assumptions:

1. The interaction is reversible; the association reaction is bimolecular while the dissociation is uni-molecular.

2. All the receptor molecules are equivalent and independent

3. The biological response is proportional to the number of occupied receptor sites.

4. The interaction and response are measured after the reaction has reached equilibrium.

5. The active chemical agent does not undergo degradation or participate in other reactions, and only exists in either a free (L_F; unbound) form or bound to the receptor (B).

Under these assumptions, at equilibrium:

$$\textbf{Equation 7.1: Receptor}_{\textbf{free}} + \textbf{Ligand}_{\textbf{free}} \underset{\textbf{k}_{-1}}{\overset{\textbf{k}_1}{\rightleftharpoons}} \textbf{Receptor:Ligand} \overset{\textbf{k}_e}{\longrightarrow} \textbf{effect}$$
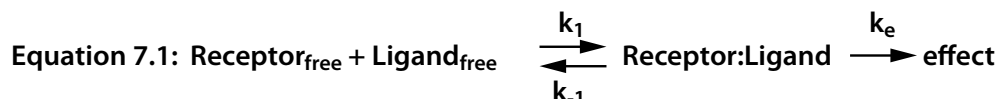
where k_1 and k_{-1} are the kinetic association and dissociation constants and k_e is the proportionality constant between response and occupancy. Because the determination of physical binding constants does not normally require the measurement of k_e, we can focus our discussion on the reversible reaction.

At equilibrium, mass action says that:

$$\textbf{Equation 7.2:} \quad \frac{[\textbf{Ligand}_{\textbf{free}}][\textbf{Receptor}_{\textbf{free}}]}{[\textbf{Receptor:Ligand}]} = \frac{[\textbf{L}_F][\textbf{R}_F]}{[\textbf{B}]} = \frac{\textbf{k}_{-1}}{\textbf{k}_1} = \textbf{K}_d$$

We can rewrite **Equation 7.2** as:

$$\textbf{Equation 7.3:} \quad \frac{[\textbf{L}_F][\textbf{R}_T - \textbf{B}]}{[\textbf{B}]} = \textbf{K}_d$$

where L_F is the free ligand concentration, R_T is the total receptor concentration, and $(R_T - B)$ is the free receptor concentration, and B is the bound ligand:receptor complex concentration. **Equation 7.3** can be rearranged to:

$$\textbf{Equation 7.4:} \quad \frac{\textbf{B}}{\textbf{R}_T} = \frac{\textbf{L}_F}{(\textbf{K}_d + \textbf{L}_F)}$$

which is the equation for a rectangular hyperbola with horizontal asymptote corresponding to 100% saturation of R_T, such that [bound] = [receptor], as shown in **Figure 7.1.** The ratio **B/R_T** is also referred to as **f**, the *fractional occupancy*. **Equation 7.4** shows that the K_d is defined as the concentration of free ligand at which 50% of the receptor sites are occupied (*i.e.,* fractional occupancy = 0.5).
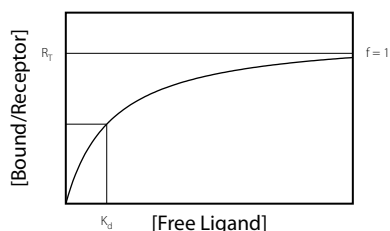
Figure 7-1. The classic hyperbolic binding curve, expressed at the fractional occupancy, f, of the receptor.

Non-specific Binding

The most common problem to deal with in receptor-ligand interactions is non-specific binding (NSB). NSB has been commonly, but incorrectly, defined as "**binding that is not saturating**" due to the presence of unlimited low affinity binding sites (*e.g.*, proteins sticking to the phospholipids of the cell membrane). Binding of the ligand would therefore be directly proportional to the concentration of **free** ligand alone. This definition has also been incorrectly stated as "**binding that was not displaceable by excess concentrations of unlabeled ligand.**" Specific binding to the receptor is routinely calculated by subtracting the measured NSB (after addition of excess unlabeled ligand to the system) from the total binding.

Since the latter definition for NSB is incorrect, results obtained using the definition above are often misleading. Binding thought to be non-displaceable and non-saturable can actually be both, if enough unlabeled or labeled ligand are added, respectively. It is better to treat NSB as binding to a set of identical and independent sites that have an affinity and capacity for the ligand in question. These sites are distinct from those of the receptor under study.

The binding of a ligand to two classes of binding sites (*e.g.*, a receptor and non-specific site) can be described by **Equation 7.5**:

$$\textbf{Equation 7.5:} \qquad B = \frac{R_1 \times L_F}{k_{d1} + L_F} + \frac{R_2 \times L_F}{k_{d2} + L_F}$$

where R_1 and R_2 are the concentrations of receptors for each site and K_{d1} and K_{d2} are the respective dissociation constants. When $K_{d2} >>> F$, **Equation 7.5** reduces to **Equation 7.6**:

$$\textbf{Equation 7.6:} \qquad B = \left(\frac{R_1 \times L_F}{R_{d1} + L_F} \right) + (K_{nsb} \times L_F)$$

The binding to the second site therefore appears linear (unsaturable). When the second site K_{d2} is very large, NSB can often be adequately described by a linear function.

When an excess of unlabeled ligand is used to estimate NSB, L_F approaches K_d and NSB will appear saturable. NSB correction of this type, with excess unlabeled ligand, results in overestimation of the number of receptor sites and underestimation of the receptor affinity. Because this overestimation increases with L_F, further artifacts can appear, incorrectly suggesting negative cooperativity or multiple binding sites.

Therefore, the best way to measure NSB would be to not to measure it at all. Rather, measure total binding and fit the data to **Equations 7.5** and **7.6** with one of the many nonlinear curve-fitting programs now available. This method requires no assumption and can account for complicated systems. This approach is especially true if NSB is in the range of <30% of total binding. If NSB is >30% of total binding, one should first attempt to experimentally reduce the percent of NSB in order to facilitate, with any confidence, the discovery of a solution. In this case, NSB is estimated as the binding that remains in the presence of 100–200 fold excess unlabeled ligand. NSB at each concentration is subtracted from total binding to arrive at specific binding. For an excellent review of NSB, see Mendel and Mendel (1985).

Determination of Binding Constants

It is not our aim to describe the details of performing a binding experiment, but a few issues should be addressed in the experimental design.

Equilibrium time: Analysis of equilibrium binding experiments assumes you measure binding at equilibrium. Before any equilibrium experiments are done, you should determine the incubation time that will allow the system to approach equilibrium. This is usually accomplished by incubating a low concentration of ligand (well below the presumed K_d) with receptor and following the amount of ligand bound over time. The incubation time for most assays is the time required for 90% of the ligand to bind **(Figure 7-2)**.

Figure 7-2. Representation of the time course for a binding event to reach equilibrium. Note that there is very little increase in binding after very long incubations.
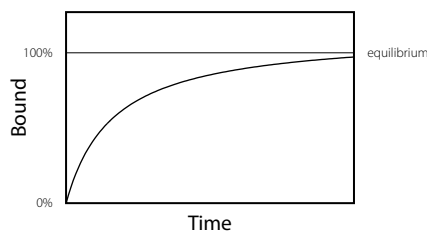
Equilibrium binding experiment: The concentration of one of the binding agents (usually the receptor) is kept constant and below the K_d ($R/K_d < 0.1$ or lower). Higher receptor concentrations tend to bind significant amounts of ligand leading to ligand depletion (discussed further in the section on **Free Ligand Concentration**).

The concentration of the other binding agent (usually the ligand) is varied from at least 100-fold below the K_d to 100-fold above the K_d. In this case, 99% of the fractional occupancy is covered by four orders of magnitude of free ligand concentration (two orders above and two orders below the K_d). In practical terms, binding experiments normally need to cover free ligand concentrations over three to four orders of magnitude (we usually aim for 20-fold below to 50-fold above the K_d). The number of points needed for analysis of the binding isotherms is dependent on the number of binding constants that need to be estimated. In general, simple models will require 15–20 single data points. Models with second binding sites and non-specific binding will require more in order to statistically analyze the data properly.

• Mendel, C.M. and Mendel, D.B. (1985) Biochem.-J. 228:269-72.

Free Ligand Concentration: Equation 7.4 describes equilibrium in terms of the free ligand concentration, not the total ligand concentration. In many experimental systems, the amount of bound ligand is a very small percentage of the total ligand concentration, and the total ligand concentration can be used as an approximation of the free ligand concentration. Generally, if the bound ligand concentration is 5% or 10% of the total ligand concentration, the approximation holds.

If the total concentration cannot be used as an approximation of the free concentration (a situation usually referred to as ligand depletion), there are a few possible alternatives. These include:

1. Accounting for the discrepancy in the a more complex binding model (See Swillens, 1995, and Kenakin, 1993)

2. Changing the experiment so that less ligand is bound (in traditional experiments, this usually means reducing the receptor concentration which results in a concomitant decrease in the bound ligand signal)

3. Measuring the concentration of free ligand in each sample by either measuring the free ligand concentration directly or subtracting the bound ligand concentration from the total ligand concentration.

Swillens (1995) and Motulsky (1995) argue that the last method, while traditionally the most popular (*i.e.,* determining the free concentration by subtracting bound from total) has problems. Specifically, the bound and new free terms will be related (as will their errors), and certain calculations of non-specific binding are impossible. Instead, the explicit solution of the binding curve, with the bound and total values in place, is recommended instead. Rearranging **Equation 7.3** and substituting (L_T - B) for L_F leads to:

$$\textbf{Equation 7.7:} \qquad \frac{R_T \times (L_T - B)}{K_d + (L_T - B)} = B$$

Solving for B, one real solution for the quadratic is:

$$\textbf{Equation 7.8: } B = \frac{(L_T + K_d + R_T) - \sqrt{(-L_T - K_d - R_T)^2 - 4L_T R_T}}{2}$$

Several software packages can automatically solve this equation for K_d and R_T.

Data analysis: Traditionally, binding data were analyzed using linear transformations of the simple binding equations. These transformations provide considerable information, but they lack the ability to analyze binding isotherms (*i.e.,* binding curves) that deviate from Clark's Theory (*e.g.,* one ligand, one receptor, no NSB). In addition, a plethora of statistical errors creep into the linear transformations, many times due to weighting effects. The most sophisticated analysis method involves the use of non-linear, least-square, curve-fitting computer programs that are capable of fitting the binding isotherms according to several different models and then compare the statistical quality of the resulting 'goodness of fit'. Many excellent Windows-based programs are commercially available.

The linear transformations are important tools in understanding binding isotherms. Here we describe one of the many step-by-step approaches to analyzing binding experiments.

- Swillens, S. (1995) *Molec. Pharm.* **47**:1197-1203.

- Kenakin, T. Pharmacologic Analysis of Drug/Receptor Interaction, 2nd Edition, 397.

- Motulsky, H. (1996). The GraphPad Guide to Analyzing Radioligand Binding Data. GraphPad Software, Inc., San Diego, CA.

Saturation Function

It is often a good idea to look at simple graphical representations of your data in order to understand what complications might be present. The simplest curve to consider is the **bound ligand vs. total ligand** curve. Note that in strict terms, the saturation function refers to the **bound receptor vs. free ligand** curve, but plotting this curve requires some calculation. The **bound vs. total** curve should be inspected for smoothness and should have no inflection points, maxima, or minima. If the curve does not level off, but continues upward linearly, it may contain an NSB component.

Klotz Plot

The most useful curve is the Klotz plot, or semi-log plot (**Figure 7-3**). Many computer analysis programs use the Klotz plot as their primary graphical representation. The binding data are plotted as bound ligand vs. log free ligand and yield a sigmoidal curve. The graphical representation of the Klotz plot is used in two ways: determining the suitability of chosen ligand concentrations and analyzing the data for cooperativity.

The Klotz plot should be symmetrical around the inflection point, which corresponds to the K_d along the free axis. The upper asymptote is R_T. The curve is nearly linear between $0.1 \times K_d$ and $10 \times K_d$. In addition, it is easy to see that 99% of occupancy is represented by four orders of magnitude of free

Figure 7-3. The classic semi-log Klotz plot. Note, that as with most binding curves, the free ligand, not the total ligand concentration, is plotted.
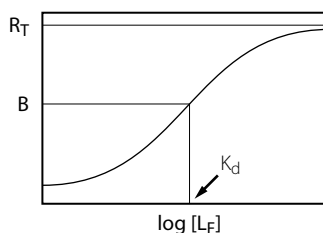
concentration (two on either side of the K_d). Lack of sufficient points on either side of the inflection point (K_d) will be obvious, especially at the plateaus. Most experiment fail because they lack data points at high ligand concentrations, due to poor design, or more often because ligand is expensive or rare. Unfortunately, fewer points in this region sometimes mean additional binding sites or non-specific binding will be missed, or the total receptor concentration (R_T) will be imprecise.

The Klotz plot can also indicate cooperativity. In the simplest model, the curve will rise from approximately 10% to 90% occupancy through $0.1X < K_d < 10X$ of free ligand concentration. If the curve completes this rise over a smaller range of concentration, this is indicative of positive cooperativity. If this rise requires a wider range of free ligand concentration, it is indicative of negative cooperativity. For a further discussion, see the section on the **Hill Plot and Cooperativity** on page 7-9.

Scatchard Analysis

Over the last thirty years, the so-called Scatchard plot has been the traditional method for analysis of binding data until the introduction of sophisticated non-linear curve-fitting software. Rearrangement of **Equation 7.3** yields:

$$\textbf{Equation 7.9:} \qquad \frac{B}{L_F} = \frac{-B}{K_d} + \frac{R_T}{K_d}$$

which fits the equation of a line (y = mx + b; B/L_F vs. B) where R_T/K_d is the y-intercept, R_T is the x-intercept, and $-1/K_d$ is the slope (see **Figure 7.4**). While many authors have written extensively on the errors inherent to analysis of binding curves by the Scatchard plot, it is a very powerful tool for identifying deviations from Clark's simple model, which without deviations, is represented by a straight line on the Scatchard plot.
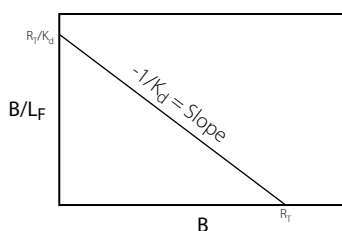
Figure 7-4. The Scatchard Curve. This representation is very sensitive to deviations from a simple binding model.

Non-specific Binding

A concave-up curve with an x-asymptote may indicate the presence of NSB (**Figure 7-5**). At first glance, it appears easy to estimate the NSB asymptote, but the mathematical subtraction of NSB from the total binding data is tedious (*i.e.,* NSB must be subtracted *radially* from the Scatchard curve because B/L_F and B are correlated, pursuant with Rosenthal's Construction). NSB in this case illustrates why computer analysis is preferred over the daunting task of **properly** deconvoluting the Scatchard plot (*i.e.,* mathematically divide it into its constituent curves).
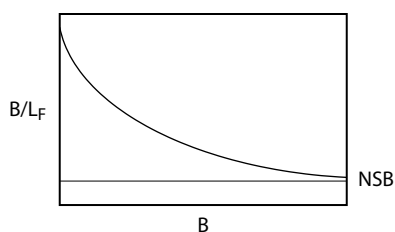
Figure 7-5. The Scatchard curve indicating the presence of non-specific binding.

Negative Cooperativity

A concave-up curve that intersects the y-axis and the x-axis may indicate the presence of negative cooperativity between receptors (**Figure 7-6**). It is often difficult to differentiate between negative cooperativity and NSB on the Scatchard plot. One suspects negative cooperativity only when other data support the possibility.
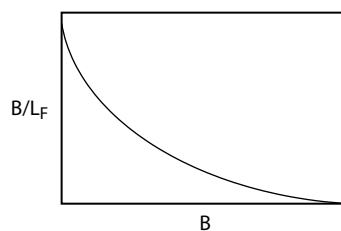
Positive Cooperativity

A concave-down curve that intersects the origin is indicative of positive cooperativity (**Figure 7-7**). The maxima occurs at: $f = (n-1)/n$, where n is the slope of the Hill plot (explained on page 7-10) and $f =$ fractional occupancy.
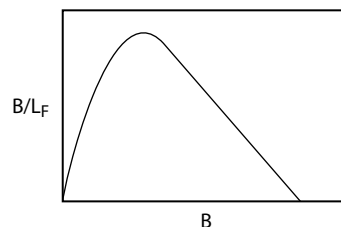
Chemical Instability at Low Concentrations

A concave-down curve that intersects the y-axis is usually due to breakdown of the ligand at low concentrations and not positive cooperativity (**Figure 7-8**). It is difficult to differentiate between these two possibilities.
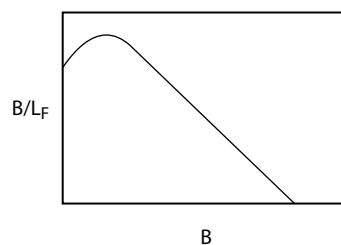
Multiple Classes of Binding Sites

A concave-up curve may also indicate the presence of multiple classes of binding sites with differing K_d values, instead of negative cooperativity or NSB (**Figure 7-9**).
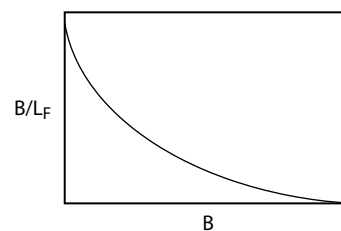
Hill Plot and Cooperativity

Many times we observe, especially with multimeric proteins, that the occupancy of some of the sites affects the affinity of the ligand for unfilled sites. The classic example is O_2-hemoglobin. The Hill Slope analysis allows for the differentiation of cooperativity (*i.e.,* when the Klotz plot (**Figure 7-3**) is steeper or shallower than predicted by Clark's model) and multiple binding sites. The Hill equation accounts for the possibility that not all receptor sites are independent and says that:

Equation 7.10: $$f_{\text{(the fractional occupancy)}} = \frac{L_F{}^n}{K_d + L_F{}^n}$$

where n is the slope of the Hill plot and is also the average number of interacting sites.

The linear transformation that is commonly used, the Hill plot, is made by rearranging **Equation 7.8** and taking the log:

Equation 7.11: $$\log\left[\frac{B}{R_T - B}\right] = n[\log(L_F)] - \log(K_d)$$

This equation is plotted as log $B/(R_T-B)$ vs. log L_F where the y-intercept is -log K_d and the slope n = the Hill coefficient (see **Figure 7-10**).
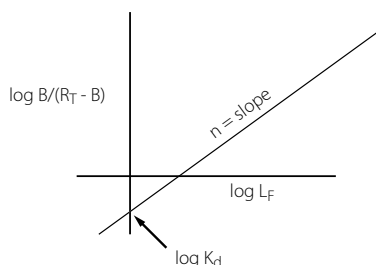
Figure 7-10. The classic Hill plot.

Please note that if the Hill plot is not linear, the model used is not applicable to the data set and needs reevaluation.

Deviations from a slope of 1 tell us about deviation from the ideal model. **Table 7-1** shows the Hill slope result and the effect on other transformations.

Table 7-1. Hill Slope Effects.			
Hill Slope	B vs. L_F plot	Scatchard	Klotz
If slope = 1, there is a single class of binding sites	hyperbola	straight line	sigmoidal
If slope ≠ 1 and is an integer, multiple non-interacting binding sites	hyperbola	concave-down	sigmoidal
If slope ≠ 1 and is fractional, you have cooperativity:			
If slope >1, Positive cooperativity	sigmoidal	concave-down	narrow sigmoidal
If slope <1, Negative cooperativity	no effect	concave-up	wide sigmoidal

One of the most elegant methods to confirm negative cooperativity was developed by De Meyts (1976). It is based on the observation that with negative cooperativity, the decrease in affinity as percent saturation increases is due to a decrease in the rate of association of the complex (net drop in K_a, the equilibrium association constant). To put it more simply, K_d varies with the percent saturation.

Experimentally, De Meyts (1976) allowed the labeled ligand to bind to its receptor until equilibrium was reached. The remaining free ligand is removed, replaced with buffer, and the amount of ligand which remains bound to the receptor is measured over time. In an identical experiment, free ligand is replaced with buffer containing a large excess of unlabeled ligand. Both data sets are plotted as B_t (*i.e.,* bound at time t)/B_o (*i.e.,* bound at time zero) vs. time. If the addition of the unlabeled ligand leads to an increase in the dissociation rate (compared to the dissociation in the absence of excess unlabeled ligand), negative cooperativity is indicated.

The issue of describing the K_d for negative cooperativity among receptor sites is addressed below. Traditionally, three parameters are reviewed:

$K_{average}$ = average affinity value (it varies with R and L_F)

K_e = maximum value of K_d when virtually all the sites are empty

K_f = minimum value of K_d when virtually all the sites are full

K_e and K_f can be estimated from the concave-up Scatchard plot. K_e is calculated as the slope of the line running from the y-intercept to the x-intercept. K_f is the slope of the line tangent to the curve at the x-intercept. $K_{average}$ varies all along the curve depending on the ligand concentration chosen.

Non-linear, Least-Squares, Curve Fitting

As with most computer programs, the axiom 'garbage in, garbage out' rings true during the analysis of binding curve data. Too often, this type of analysis is done without careful thought to what the data are telling you about deviations in your system from the ideal. Careful inspection of the data, using the methods described above, will allow you to most suitably fit your data to a model that accounts for the physical reality. If you see NSB, the computer programs will allow you to subtract it out. If you might have one receptor site or two, the programs will allow you to reanalyze your data with several models and compare them to each other with statistical robustness. People regularly report the K_d from a computer analysis and never take the time to see if the fitted curve actually fits the data well.

In general, the model that is chosen to explain a particular system should be the simplest and most statistically significant one. For example, if the data fit a model with two receptor sites no better than a model with one receptor site, the one receptor model should be chosen, unless other independent data suggest using the two-site model.

• De Meyts, P., Bianco, A.R. and Roth, J. (1976) *J. Biol. Chem.* **251**:1877-88.

Multiple Classes of Binding Sites

A common situation occurs with the presence of multiple classes of binding sites in a receptor preparation. The second site may be another high-affinity receptor or may be a low-affinity site more akin to NSB. It is important to properly identify multiple sites (see **Table 7-2**).

Table 7-2. Characteristics of Multiple Binding Sites.	
Plots	Identifying Feature
B vs. LF plot	A polynomial of n + 1 degrees being the sum of two hyperbolas
Scatchard plot	Concave-up curve (Figure 7-7)
Hill plot	Slope not equal to 1
Klotz plot	Multiple inflection points if the K_d values differ enough

The changes in the Klotz plot may be very small. Usually, the ratio of the K_d values must be at least 100X before you will see a second inflection point. If there is a <14-fold difference in the K_d values, you will not be able to mathematically deconvolute the sites. On the traditional B vs. L_F plot, the composite curve will appear to be a normal hyperbola, but the apparent K_d is actually equal to $[(K_{d1})(K_{d2})]^{1/2}$. For this reason, the Scatchard plot, which normally shows a straight line, is the easiest way to see multiple classes of binding sites because it will be concave-up when multiple sites are present. Note that the multi-site Scatchard curve is the sum of two (or more) linear lines with different intercepts and slopes. The K_d of a line forced through the total curve does not correspond directly to the K_d of either site. This relationship is presented mathematically in **Equation 7-12**:

$$\text{Equation 7.12:} \quad B = \frac{R_{T1} \times L_F}{K_{d1} + L_F} + \frac{R_{T2} \times L_F}{K_{d2} + L_F}$$

This model can be complicated more by the addition of a linear NSB term (resulting in a total of five parameters: R_{T1}, K_{d1}, R_{T2}, K_{d2}, NSB). Models of more than five parameters are difficult to analyze. At least 80 data points are required to tell the difference between a five and seven parameter model. Generally, multiple sites are confirmed by fitting the data to a one-site (± NSB) model and two-site (± NSB) model, and then determining which model gives a statistically better fit.

Many times, it is not clear whether to call a low affinity second site a true binding site or non-specific binding. This issue was addressed earlier in the chapter. As discussed there, if the experiment covers a concentration range such that $K_{d2} \ggg L_F$, then the second site will collapse into a NSB component.

Competition in Binding

The term "competition," as used in this section, refers to the special case of antagonism in which two ligands are capable of binding to the same sites. The simplest case is the binding of a labeled ligand in the presence of various concentrations of an unlabeled ligand (also called the inhibitor or competitor) to a receptor with one class of binding sites. Competition experiments are useful for a few reasons:

Determining whether the labeling process has affected the ligand's affinity for the receptor. The labeled ligand's K_d determined by the direct binding experiment is compared to the K_i values of the unlabeled ligand and mock-labeled ligand that were determined from the competition assay. If the K_i and K_d value are not the same (within the error of the assay—say, four-fold) then the labeling process may have affected the affinity.

Comparing the affinities of several ligands for the same receptor. It is more reliable to compare the affinities of several ligands when none are labeled. A single control labeled ligand is used for comparison to each unlabeled ligand. The control ligand may be of absolutely no interest itself. Each unlabeled ligand will generate its own binding isotherm, and as long as the conditions of the experiments are identical, the affinities of each can be directly compared. The advantage of this approach is that since only a single ligand is labeled, adverse effects on affinity caused by the labeling process will not affect the comparison of the unlabeled ligands.

The IC_{50} is the concentration of inhibitor necessary to displace 50% of the labeled ligand. It is a useful binding constant to characterize the inhibitory ligand (See **Figure 7-11**). If your aim is to directly compare the relative affinities of several inhibitors, comparing IC_{50} values obtained under identical conditions is sufficient.

Figure 7-11. The standard competition curve. Please not that the IC_{50} is not necessarily equal to the KI.
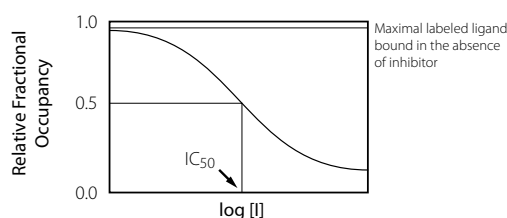
Historically, the affinity of the inhibitor for the receptor has been derived using the Cheng and Prussoff (1973) Corrections:

$$\textbf{Equation 7.13:} \qquad f_i = \frac{I}{I + K_i(1 + L_T/K_d)}$$

and

$$\textbf{Equation 7.14:} \qquad f = \frac{L_T}{K_d(1 + I/K_i) + L_T}$$

where: f_i = the fractional inhibition

f = fractional occupancy or saturation

L_T = total concentration of the labeled ligand

I = Added concentration of the unlabeled ligand (competitor/inhibitor)

K_d = dissociation constant for labeled ligand

K_i = dissociation constant for the unlabeled ligand

•   Cheng, Y.C. and Prusoff, W.H. (1973)
Biochem. Pharm. **22**:3099-108.

When the value of f_i or f is equal to 0.5, **Equations 7-9** and **7-10** can be simplified to:

$$\text{Equation 7.15:} \qquad K_i = \frac{IC_{50}}{1 + L_T/K_d}$$

and

$$\text{Equation 7.16:} \qquad K_d = \frac{EC_{50}}{1 + I/K_i}$$

where EC_{50} is the concentration of ligand that yields 50% binding in the presence of a given concentration of inhibitor.

Total vs. free for the labeled ligand and unlabeled inhibitor. Equations 7.15 and **7.16** were originally derived in the context of competitive enzyme inhibition. In the original equation, the concentrations of L would be the **free** concentration (not initial or total) of labeled ligand and the IC_{50} would be the **free** concentration (not added or total) of inhibitor that reduces the binding of the labeled ligand by 50%. However, enzyme kinetic studies are usually done under conditions such that the free and total concentrations of substrate and the free and total concentrations inhibitor are nearly identical (*i.e.,* [Total] approximates [Free]). This may not be true of equilibrium binding studies, and therefore the total concentrations cannot be substituted for free concentrations without the possibility of introducing significant errors into the calculation of K_i (Hollenberg and Cuatrecasas, 1979). Several authors have approached this issue (see Rodbard, 1973; Jacobs *et al.,* 1975; Kenakin, 1993; Munson and Rodbard, 1988). These authors show quite dramatically how high receptor and labeled ligand concentrations lead to an error in the IC_{50}, and their papers are essential reading for anyone performing competitive displacement experiments.

Munson and Rodbard (1988) offered an exact solution (**Equation 7-17**) of the Cheng and Prusoff Correction that takes these possible problems into account.

$$\text{Equation 7.17:} \qquad K_i = \frac{IC_{50}}{1 + \dfrac{L_T(y_0 + 2)}{2 \times K_d(y_0 + 1)} + y_0} - K_d\left(\frac{y_0}{y_0 + 2}\right)$$

where y_0 = the initial Bound to Free ratio for the labeled species before perturbation of equilibrium by the added inhibitor and IC_{50} = the concentration of inhibitor that reduces binding of the labeled ligand by 50%.

When y_0 is very small, **Equation 7-17** reduces to **Equation 7-15**. Generally, if y_0 is <0.1, the problem can be ignored.

An alternative approach is to calculate the exact solution of **Equations 7.13** and **7.14** in terms of free ligand and inhibitor concentrations (*i.e.,* Free = Total - Bound). Kenakin (1993; also see Swillens, 1995) offers the following solution (**Equation 7-18**) and shows that if receptor concentration is 10 times the K_d, the IC_{50} will overestimate the K_i by about 10-fold. It should be noted that all errors caused by high receptor and ligand concentrations lead to overestimations of the K_i by the IC_{50}.

$$\text{Equation 7.18:} \qquad B = \frac{(L_T \times R_T) - (B \times R_T)}{L_T - B + K_d + (I \times K_d/K_i)}$$

• Hollenberg, M.D. and Cuatrecasas, P. (1979) The receptors: a comprehensive treatise. New York:Plenum Press.

• Rodbard, D. (1973) Receptors for reproductive hormones. New York: Plenum Press.

• Jacobs, S. *et al.* (1975) *Biochem. Biophys. Res. Commun.* **66**:687.

• Kenakin, T. P. (1993) Pharmacologic analysis of drug/receptor interaction, 2nd ed. New York:Raven. 483 p.

• Munson, P.J. and Rodbard, D. (1988) *J. Receptor Res.* **8**:533-46.

• Swillens, S. (1995) *Molec. Pharm.* **47**:1197-1203.

Rearranging to solve for K_i:

Equation 7.19:
$$K_i = \frac{B \times I \times K_d}{(L_T \times R_T) + B \times (-R_T - L_T + B - K_d)}$$

In order to convert the observed IC_{50} to K_i, solve this equation for conditions when the added inhibitor concentration, I, equals the IC_{50}. If your model for competitive binding includes a non-specific binding term, Swillens suggests how this second method can be modified to account for deviations to NSB by high receptor and ligand concentrations.

Equilibrium and Non-equilibrium Conditions

Equilibrium Conditions

One of the central assumptions to the analysis of binding data is that the data are gathered after equilibrium between the receptor and ligand is reached. This may take minutes or several hours. Before meaningful binding experiments can be done, the incubation time necessary to reach equilibrium must be determined. Usually this is accomplished by following the time course of binding at a single ligand and receptor concentration (see **Figure 7-12**). Because equilibrium is reached more slowly when concentrations are low, choose the lowest concentration of ligand possible for the time course experiment. Binding experiments are then usually done at a time when >90% of equilibrium is reached.

Figure 7-12. A graphical representation of an experiment used to determine the time required for equilibrium binding to occur.
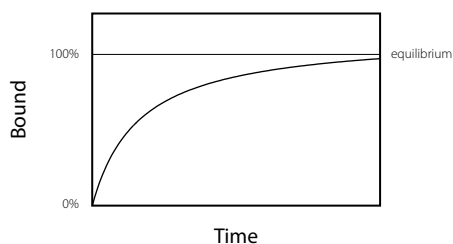
Non-equilibrium Conditions

There are varieties of reasons why researchers may be interested in obtaining measurements of bound ligand versus time, under non-equilibrium conditions. For instance, the receptor/ligand K_d may be so low that concentrations of labeled ligand required in an equilibrium binding experiment might be lost in the background. The only way to calculate binding constants may be through kinetic experiments.

Determination of the Association Rate Constant, k_1

Introduction. Clark's model can be written as:

Equation 7.20:
$$(R_T - B) + (L_T - B) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B$$
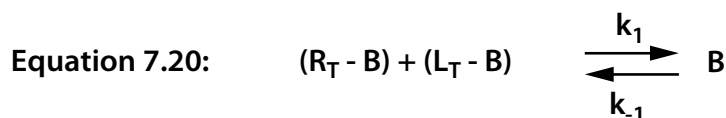
Where R_T is the total receptor concentration, $(R_T - B)$ is the free receptor concentration, L_T is equal to the total ligand concentration and $(L_T - B)$ is equal to L_F, the free ligand concentration. During a very small time interval, δt, the concentration of bound ligand (*i.e.,* receptor:ligand complex) will change by a small amount, δB_t, and therefore the following equation applies:

Equation 7.21: $$\frac{\delta B_t}{\delta t} = k_1(R_T - B_t)(L_T - B_t) - (k_{-1} \times B_t)$$

At equilibrium, $\delta B_t/\delta t = 0$ and the equation can be rewritten as a quadratic, with one meaningless solution, B', and a real solution, B_e. Bound, at any time t can then be written as **Equation 7.22**:

Equation 7.22: $$B_t = B_e + \frac{(B' - B_e)}{1 = e^{(mt + C)}}$$

where $m = (k_i)(B' - B_e)$, C (the integration constant) $= \ln(B'/B_e)$, and B_e is the amount bound at equilibrium. When $t = 0$, $B_t = 0$, and at infinite time, B_t approaches B_e, the real solution (see **Figure 7-12**).

Solution of k_1. Solving **Equation 7.22** by non-linear regression analysis, using the time-dependent binding data at a single ligand concentration, will yield the four parameters of this equation: B_e, B', m, and C. If the experiment is repeated n times, at n different ligand concentrations, n sets of parameters will be obtained. From these parameters, obtained at many ligand concentrations, k_i is determined by plotting the n values of m obtained with n different ligand concentrations versus the respective values of $(B' - B_e)$. This plot will generate a straight line passing through the origin, with a slope of k_i.

Calculation of the dissociation constant.

Equation 7.23: $$B_e + B' = R_T + L_F + \left(\frac{k_{-1}}{k_1}\right)$$

Plotting the n values of $(B_e + B' - R_T)$ versus the n values of L_F should yield a straight line with a slope of 1 and a y-intercept of K_d. Knowing the k_i and K_d, k_{-1} can be calculated as $k_{-1} = (K_d)(k_i)$.

Independent Determination of the Dissociation Rate Constant, k_{-1}

The determination of the dissociation rate constant, k_{-1}, of a labeled ligand from its receptor is commonly accomplished by allowing the labeled ligand to bind to the receptor until equilibrium is reached and then measuring the rate of dissociation of ligand from receptor. In solid-phase binding experiments, labeled ligand is allowed to bind to immobilized receptor and then the incubation medium is quickly removed from the dish or filter, and replaced by buffer. This step instantaneously removes any free ligand from the system (hence the term "instantaneous dilution"). As the bound ligand reaches a new equilibrium with the ligand in the buffer (zero ligand in solution at time = 0), the amount of ligand bound to receptor will decrease over time, as shown by the **Equation 7-24**:

where B_t is the amount of ligand bound at any time t, and B_0 is the total ligand bound at time zero.

Equation 7.24: $$B_t = B_0 \times [e^{(-k_{-1} \times t)}]$$

When $B_t = 0.5\ B_0$, then $k_{-1} = 0.693/T_{1/2}$ where $T_{1/2}$ is the half-time of dissociation (see **Figure 7-13**).

Figure 7-13. The dissociation binding curve.
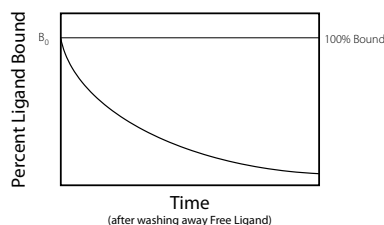
$T_{1/2}$ is measured from a plot of ln (B_t/B_0) vs. t, and is the time when ln $(B_t/B_0) = 0.5$ (**Figure 7-14**).

Figure 7-14. The graphical method of determining $T_{1/2}$.
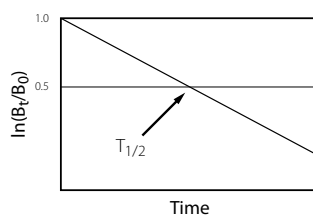
This method for determining the dissociation constant will yield reliable results unless the system exhibits positive or negative cooperativity in binding.

Approximate Solutions for k_1 and k_{-1}

The complication of non-linear regression analysis of the association curves in order to calculate k_1 and k_{-1} can be avoided if one assumes that the amount bound is much less than the amount free (B <<< L_F), as is the case in many binding experiments. If B <<< L_F, then **Equation 7.21** can be simplified, and its integral yields **Equation 7.25**:

$$\textbf{Equation 7.25:} \qquad B_t = B_e \times \{1 - e^{[-(k_1 R_T + k_{-1})t]}\}$$

When $B_t = 0.5 B_e$, then **Equation 7.25** can be transformed to **Equation 7.26**:

$$\textbf{Equation 7.26:} \qquad \frac{\ln(2)}{T_{1/2}} = k_1 L_T + k_{-1}$$

where $T_{1/2}$ is the half-time of association (between ligand and receptor). The rate constants are therefore calculated in the following way:

1. Repeat an association binding experiment at several different ligand concentrations, collecting data on bound vs. time.
2. Analyze each curve for B_e, the amount bound when equilibrium is reached.
3. Plot the association data from each experiment as log $(B_e - B_t)/B_e$ vs. time.
4. Determine the $T_{1/2}$ for each curve (point at which log $(B_e - B_t)/B_e = 0.5$).
5. Plot ln(2)/$T_{1/2}$ vs. LT. According to **Equation 7.26**, this plot will yield a straight line with slope $=k_1$ and y-intercept $=k_{-1}$.

Of course, you could always use the kinetic subroutines of the commercially available curve-fitting programs to help in many of these calculations.

Ways to Improve Experimental Results

Interchangeability of Terms

While it is common to describe the analysis of binding with the terms "receptor" and "ligand," it is important to note that these terms are actually interchangeable. There is no reason why the "receptor" can't vary in concentration while the ligand concentration remains constant. These terms can also apply to a variety of systems. For instance, replace these terms with "antibody" and "antigen" and all of the same rules apply. In reviewing the issues discussed throughout **Chapter 7**, it is important to keep in mind how the guidelines on the analysis of binding data can be applied to specific, individual circumstances. In **Chapter 8**, specific examples using fluorescence polarization are discussed.

Important Points

- Upward concavity in the Scatchard plot may result not only from multiple classes of sites but from negative cooperativity or from experimental artifacts.

- Although experimental points might appear to correspond to a curvilinear Scatchard plot, it is important to determine whether or not a straight line is an equally adequate fit.

- The most common experimental artifact results from incorrect estimation of free ligand concentration due to inadequate separation of the bound ligand. This may also result in neglecting NSB.

- If the receptor is more stable with bound ligand than without, the effective concentration of receptor, R, decreases as bound receptor:ligand, B, diminishes; this may result in apparent positive cooperativity.

- Binding curves will have meaning only if measurements are done under equilibrium conditions.

- Do not average replicate values. It decreases your degrees of freedom. In addition, it is better to run more individual concentrations, than fewer ones with replicates.

Common Binding Experiment User Errors

- Neglecting a proper correction for NSB.

- Determination of NSB by competition with excess unlabeled ligand. If the unlabeled ligand is present in excessive amounts, correction by simple subtraction may be misleading.

- Pooling data from separate experiments with different protein concentrations. This cannot be done unless the bound and bound/free values are normalized (dividing by the protein concentration).

- Presence of a non-binding contaminant in the labeled ligand. Since the contaminant is not bound, its signal will be computed as part of free ligand.

- Not taking into account different physical and/or chemical properties of the labeled and the unlabeled ligand with respect to the interaction with binding sites.

- Inadequate number or range of ligand concentrations (*i.e.,* only a 100-fold range of concentrations).

- Not taking into account internalization of the ligand/receptor complex or its degradation in experiments involving cells.

Acknowledgment

Analysis of FP Binding Data

Determination of Binding Constants

Definitions

"**Receptor**" and "**ligand**" have traditionally been used to describe large, membrane-bound receptors and small, soluble ligands, respectively. In fact, the terms are interchangeable when discussing any two molecules that bind to each other; the analysis of binding data does not draw a distinction between the two terms. In most traditional binding experiments, the receptor concentration is kept constant and the ligand concentration is varied. It is equally correct to keep the ligand concentration constant and vary the receptor concentration. In FP, on the other hand, it is the small, labeled ligand that is held constant at low concentrations and a much larger, unlabeled molecule is titrated against it. The experiments are done this way in order to vary the labeled ligand from the completely free state (and lowest polarization value) to the completely bound state (and highest polarization value, thus maximizing the dynamic range of the experiment. The role of the receptor and ligand in FP experiments is opposite that of traditional radioligand experiments (where [receptor] is held constant and [ligand] varies). The scientist using fluorescence polarization must, therefore, decide whether to use the classic equations as written and hope to remember that receptor really means ligand (and *vice versa*), or instead rewrite those equations for experiments using fluorescence polarization.

In past editions of this Guide, we had chosen the former route—to the endless confusion of our readers. In this edition, we have taken then second route, rederiving these equations specifically for FP-based applications. We still retain the intuitive phrases "ligand" to refer to the small, labeled molecule, and "receptor" for the much larger, unlabeled molecule.

Polarization vs. Anisotropy

Polarization and anisotropy are both derived from the measured vertical and horizontal intensities (see **Chapter 1**). The values are mathematically related and easily interconverted. Both values represent a weighted average of the bound vs. unbound states of the fluorescent molecule. If most of the ligand is unbound, the polarization/anisotropy value will be low. As the fraction of labeled molecules that is bound increases, the polarization/anisotropy value increases to a maximum value that corresponds to the fluorescent molecule being 100% bound.

A population of excited, identical molecules in solution all have the same polarization value. If a portion of these molecules undergo an apparent size change such that their rotational relaxation rate also changes, the observed polarization value represents an average of the component polarizations of all of the molecules. The polarization of a mixture of molecules is given by Weber (1952):

$$\textbf{Equation 8.1:} \qquad \left(\frac{1}{P} - \frac{1}{3}\right)^{-1} = \sum_{i=1}^{n} f_i \left(\frac{1}{P_i} - \frac{1}{3}\right)^{-1}$$

where each fluorophore species has a polarization P_i and fractional fluorescence intensity f_i. On the other hand, the additivity of anisotropies is given by a simpler equation:

$$\textbf{Equation 8.2:} \qquad A = \sum_{i=1}^{n} (f_i)(A_i)$$

• Weber, G. (1952) *Biochem.* **51**:145-55.

Due to mathematical simplicity, anisotropy values are preferred because it is easier to deconvolute anisotropy values into their component values than it is polarization values. It should be noted again that in the majority of applications, anisotropy does not give any additional information than polarization.

As shown in **Table 8-1**, polarization values can be manipulated as if they were anisotropy values (*i.e.,* combined by simple additivity); the resultant errors are in the range of less than 1% to 2.5% when polarization values reach 250 mP.

Table 8-1. The polarization value of a hypothetical mixture was calculated using Equation 8.1 by treating the polarization values as though they were anisotropies (simply additive).

		Fraction	Using Equation 8.3	Using Simple Additivity	Error
Example 1					
Free Ligand	50 mP	80%			
			71 mP	70 mP	<1%
Bound Ligand	150 mP	20%			
Example 2					
Free Ligand	50 mP	60%			
			133 mP	130 mP	2.5%
Bound Ligand	250 mP	40%			

Since most instruments calculate both polarization and anisotropy, there is little reason to use polarization in your analysis. If your instrument does not report anisotropy values, the two are easily interconverted:

$$\text{Equation 8.3:} \qquad A = \frac{2 \times P}{3 - P}$$

We generally use the term "Fluorescence Polarization" instead of "Fluorescence Anisotropy" because FP is most often the term used to describe the entire technology. In many applications that involve a minimum of curve analysis, we still use polarization.

Relationship of Anisotropy to Bound/Free Ratio

Fluorescent molecules involved in binding events (*e.g.*, ligand-receptor interactions) will exist in only one of two states: bound or free. In the general case, the bound and free states of the fluorescent ligand will each have a unique anisotropy value—high for the bound state and low for the free state (quenching caused by binding complicates the analysis). With only two species, the anisotropy additivity equation reduces to:

$$\text{Equation 8.4:} \qquad A = F_f A_f + F_b A_b$$

where: $F_f + F_b = 1$

A = observed anisotropy value

F_f = fraction of fluorescent ligand that is free

F_b = fraction of fluorescent ligand that is bound

A_f = anisotropy of the free fluorescent ligand

A_b = anisotropy of the bound fluorescent ligand

The top and bottom plateaus of the semi-log equilibrium binding isotherm (anisotropy vs. log total receptor concentration) define the anisotropy of the free and bound states, A_f and A_b. With the observed anisotropy, A, we can calculate the fraction of bound and free fluorescent ligand for a given anisotropy value. For instance, if the A_{free} = 60 mA (mA = millianisotropy units) and A_{bound} = 160 mA, at a mA = 110, half of the fluorescent ligands will be bound and half will be free. At A = 135 mA, 75% of the fluorescent ligands will be bound. This linearity of response means that anisotropy values can be equated directly to 'fraction bound,' though it may be more prudent to convert anisotropy values to bound ligand concentration. This is especially true for the proper analysis of direct equilibrium binding experiments, when the value of bound ligand is required to calculate the concentration of bound and free unlabeled receptor.

Changes in Fluorescence Intensity and Fluorescence Lifetime

The simple use of polarization or anisotropy data is predicated on the quantum yield of the fluorophore being the same in the bound (Q_b) and free (Q_f) state. Changes in quantum yield of the fluor is usually not a problem unless the fluor is directly involved in the binding event, for instance, with the binding of fluorescein by a anti-fluorescein antibody. **Chapter 3** describes the mathematical corrections necessary when quenching occurs.

Seeing a Signal

In order to see a change in polarization, the concentrations of ligand and receptor concentrations must be chosen so that at low receptor concentrations, the ligand remains unbound (low polarization value), and at high receptor concentrations, the ligand is primarily bound (high polarization value). Careful choice of the experimental concentrations will maximize the difference between the highest and lowest polarization values and therefore increase the sensitivity of the assay.

Solving the general **Equation 7.2** for B (receptor:ligand complex) in terms of R_T and L_T yielded **Equation 7.8**, presented in this chapter as **Equation 8.5**:

$$\textbf{Equation 8.5:} \qquad B = \frac{L_T + K_d + R_T - \sqrt{[(L_T + K_d + R_T)^2 - 4L_T R_T]}}{2}$$

Notice that **Equation 8.5** is perfectly symmetrical with respect to R and L, as stated at the beginning of this chapter. That is, R_T and L_T are interchangeable. Experimentally, either the ligand or receptor can be held constant and the other varied.

The explicit solution of B in terms of R_T and L_T, and the obvious interexchangeability of R_T and L_T is a striking demonstration that the analysis of FP data is completely analogous to the analysis of classical radioligand binding experiments. The implications of **Equation 8.5** with regards to the percentage of ligand and receptor bound at different concentrations are shown in **Table 8-2**.

Table 8-2. Range of percentages of ligand and receptor bound under typical experimental conditions.			
Ligand Concentration ($[L]/K_d$)	Receptor Concentration ($[L]/K_d$)	% Ligand Bound at Equilibrium	% Receptor Bound at Equilibrium
0.01	0.01	1	1.0
	0.1	9	0.5
	1	50	0.1
	10	91	0.09
	50	98	0.02
0.1	0.01	0.9	9.0
	0.1	8.4	8.4
	1	49	4.9
	10	91	0.9
	50	98	0.2
1	0.01	0.1	50
	0.1	5.0	49
	1	38	38
	10	90	9.0
	50	98	2.0
10	0.01	0.1	91
	0.1	0.9	90.8
	1	9	90.1
	10	73	73
	50	98	19.5

We would normally design FP binding experiments so that the labeled ligand concentration would be kept well below the K_d ($[L]/K_d < 0.1$). As receptor is titrated against ligand, the percentage of ligand bound varies across a wide percentage (*i.e.,* large signal dynamic range). Simultaneously, very little receptor is bound, thus avoiding a situation of "receptor depletion" (analogous to the "ligand depletion" described in **Chapter 7**).

If the FP experiment is run at a higher constant ligand concentration, as occurs in many cases, the percent of the ligand bound versus receptor concentration still covers an adequate range, but significant amounts of receptor are also bound ("receptor depletion"). In this case, the simplified binding equations, which assume only a tiny fraction of receptor is bound, cannot be used. The explicit equations must be used instead. The determination of K_d and the solution of binding equations must be dealt with mathematically as described in **Chapter 7** and later in **Chapter 8**. In the case of direct binding and competition experiments, unless the correct mathematical treatment is applied, the observed EC_{50} or IC_{50}, respectively, will be overestimations of the true K_d and K_i.

It is fortuitous that in FP competition experiments, since the receptor concentrations must be necessarily high to bind significant fluorescent ligand (the maximizing the polarization value), the "receptor depletion" effect is reduced (*e.g.*, [ligand] = $1 \times K_d$ and [receptor] = $10 \times K_d$, so that 90% of the ligand is bound, but only 9% of the receptor; **Table 8-2**).

Equilibrium Binding: Experimental Design

Incubation Time to Reach Equilibrium

Before a classic receptor/ligand equilibrium binding experiment can be completed, the incubation period required for equilibrium to be achieved must be empirically determined. Time to reach equilibrium is dependent on the concentrations of the ligand and receptor, so the worst case concentrations should be used, namely, the chosen constant labeled ligand concentration and the lowest receptor concentration envisioned.

Step 1.   Determination of Equilibrium Time using the Beacon® 2000 System

1. Background blank the buffer.

2. Add the ligand to the tube and take several readings to determine the polarization of free ligand.

3. To the same tube, add an amount of receptor approximately equal to 1/20 of the expected K_d. Because the rate of association is dependent on the receptor concentration, this combination of low ligand and receptor concentrations represents the longest time necessary to reach equilibrium.

4. Follow the increase in polarization over time (begin with 10 minute intervals). Determine the time at which the polarization values plateau, representing the approach to equilibrium. Use this time for the incubation period of the binding experiments outlined later.

Constructing a Binding Isotherm

As described earlier, the format of the typical equilibrium FP binding experiment involves incubating a sub-K_d concentration of labeled ligand with a wide range of receptor concentrations from below to above the anticipated K_d. In those cases when there is no prior information about the K_d, it is not disastrous to incorrectly choose these concentrations; it will become apparent when the data are inspected if the experiment should be repeated with a different range of receptor concentrations.

Step 2. Construction of a Binding Isotherm Using the Beacon® 2000 Analyzer

1. Serially dilute your unlabeled receptor into approximately 20 test tubes, covering the range of concentrations from 10- to 20-fold below the K_d to approximately 50- to 100-fold above the K_d. The final volumes should be at least 0.1 mL.

2. If the background fluorescence of the tube with the highest concentration of receptor is very low, there is no need to account for the background fluorescence. The background contributed by some samples (*e.g.*, from impure protein or antibody preparations) can be high and must be taken into account. There are two suggested correction methods:

   a. If polarization values will be determined only once, a single set of tubes is sufficient (as described above). Measure and record the background intensity of each tube with the Beacon® 2000 Fluorescence Polarization System in its batch mode and then continue on to **Step 3**.

   b. In some experiments, it may be useful to determine polarization values several times, for instance, after incubations at different temperatures. Background fluorescence values need to be determined each time the samples are reread. If the labeled ligand has already been added to the tubes, measurement of background fluorescence is impossible. In these cases, prepare a duplicate set of tubes that will not receive the fluorescent ligand.

3. Add identical aliquots of the ligand to each tube and mix.

4. Incubate the tubes for the time determined in **Step 1**.

5. Measure the polarization value of each tube. Because the Beacon® 2000 Fluorescence Polarization System is being used in the batch mode, it is important that determinations are made in the same order as blank readings were taken.

Analysis of Binding Constants

Upon completion of the binding experiment, the measured data will be millipolarization (or millian-isotropy) units vs. total receptor concentration. Much was said in the previous chapter about analysis of binding curves. In this Section, we will limit our discussion to only the practical considerations of analysis of these data.

Step 3. Convert to Anisotropy

If you didn't already record your data as anisotropy, now is the time to convert the polarization values. Either acquire the anisotropy data from your downloaded Beacon® 2000 Fluorescence Polarization System spreadsheet or convert your polarization values to anisotropy.

Step 4. Receptor Depletion?

Before choosing the equations to use for analysis, it is important to know whether receptor depletion is a problem. That is, is a significant percentage of the receptor is bound such that the total receptor concentration cannot be used as a good approximation of the free receptor concentration. In determining if there is a problem, the mA values must first be converted into bound ligand concentrations using the following equation:

Equation 8.6: $[\text{Ligand Bound}] = L_T \times \dfrac{A - A_f}{A_b - A_f} = B$

where:

L_T = the total added concentration of ligand

A = the experimental anisotropy

A_f = the anisotropy for the free ligand

A_b = the anisotropy for the fully bound ligand

The quotient is termed F_b and is equal to the fraction of ligand bound. For this application, the values of A_b and A_f can be visually estimated or determined by a curve-fitting program. Later we will want precise values for these parameters.

For each receptor concentration we now have a corresponding concentration of bound ligand (really the receptor:ligand complex), and it is easy to compare these values to determine what percentage of receptor is bound in the complex. If the bound receptor:ligand complex concentration is greater than 10% of total receptor concentration, we should not substitute the total receptor concentration for the free receptor concentration.

Step 5.   Analysis of Binding Isotherm with Receptor Depletion

As stated in the previous chapter, there are several methods to correct for receptor depletion. Probably the most obvious method is to calculate the free receptor concentration by subtracting the bound receptor from total receptor since you know both of these values. Most people do this, and it is a legitimate method, but as discussed in **Chapter 7** there are problems with this approach. First, bound and free concentrations, and their errors, are related, and second, the effect of non-specific binding cannot be addressed properly (luckily, NSB is almost never present in any measurable degree in FP-based experiments, because the technique is solution-based). The intuitive method described above will work, but we encourage all readers to seek out the references mentioned in **Chapter 7** for a more complete explanation of the binding analysis in the presence of receptor depletion. The decision to calculate free receptor concentrations by subtraction or to use the explicit equations below is best left to the researcher and their unique situation.

Following the logic in **Chapter 7**, we derived the equation for the binding isotherm which can be rearranged to:

Equation 8.7: $B = \dfrac{R_F \times L_T}{K_d + R_F}$

If we choose not to estimate R_F, the free receptor concentration, with R_T, the total receptor concentration, we must substitute the term $(R_T - B)$ for R_F.

Equation 8.8: $B = \dfrac{L_T(R_T - B)}{K_d + (R_T - B)}$

One solution for B in **Equation 8.8** is:

Equation 8.9:
$$B = \frac{L_T + K_d + R_T}{2} - \frac{\sqrt{(-L_T - K_d - R_T)^2 - 4L_TR_T}}{2}$$

This is the equation Kenakin (1993) essentially gives as his Eq. 12.14, and Swillens (1995) as his Eq. 3 (with a non-specific binding component), and Lundblad *et al.* (1996) as their Eq. 8. This equation can be solved by a non-linear regression program yielding estimates for K_d and L_T. If you would rather use the anisotropy data directly without conversion to bound receptor/ligand values, substitute **Equation 8.9** for B in Equation 8.6 and solve for A, the measured anisotropy.

Equation 8.10:

$$A = A_f + (A_b - A_f) \times \frac{(L_T + K_d + R_T) - \sqrt{(L_T - K_d - R_T)^2 - 4L_TR_T]}}{2L_T}$$

L_T is known and A is measured for each R_T. The equation can now be solved for K_d, A_b, and A_f.

Step 6. Analysis of Binding Isotherm without Receptor Depletion

If receptor depletion is not a problem, these equations can be simplified because R_T is a good approximation of R_F.

Equation 8.11:
$$B = \frac{R_T \times L_T}{K_d + R_T}$$

Again, this equation can be solved by computer assisted non-linear regression if the anisotropy data are converted to bound receptor:ligand complex, B, or the anisotropy data can be fitted directly. In a manner analogous to the derivation of **Equation 8.10**, we get:

Equation 8.12:
$$A = A_f + \left[(A_b - A_f) \times \frac{R_T}{K_d + R_T}\right]$$

which will yield values for K_d, A_f, and A_b.

• Kenakin, T.P. (1993) Pharmacologic analysis of drug-receptor interaction. New York:Raven. 483 p.

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

• Swillens, S. (1995) *Mol. Pharm.* **47:**1197-1203.

Competition Experiments

General Considerations

Fluorescence Polarization competition experiments necessitate high receptor concentrations in order to bind significant amounts of the fluoresceinated tracer and therefore cause a shift in the polarization value. If the [receptor]/K_d ratio is ≤ 0.1, according to **Table 8-2** no more than 10% of the ligand will be bound, and the starting polarization value will still be only 10% of the maximum value. Any drop in polarization caused by the addition of an unlabeled competitor will be limited to only 10% of the possible dynamic range.

Instead, FP competition experiments should be designed such that the [receptor]/K_d ratio is about 1, so that the starting polarization value will represent 50% of the maximal shift (see **Table 8-2**). Indeed, we recommend choosing conditions such that the shift is approximately 80%. Under these conditions, though, more inhibitor is required to see a 50% drop in the amount of bound ligand compared to when the [receptor]/K_d = 0.1. The result is that the observed IC_{50} will be an overestimation of the true K_i.

Receptor-Ligand Competition Experiments

Step 1.   Determine the Minimum Incubation Time Necessary to Reach Equilibrium.

Presumably, this information was determined previously for the direct binding experiment. If not, some estimate of the time needed to reach equilibrium is required. Ideally, a competition experiment using a low concentration of competitor can be followed over time (reading the set of tubes at several different times until polarization values plateau).

Step 2.   Constructing a Competition Isotherm

1.   Serially dilute the unlabeled competitors over a range of concentrations, with a total volume for each tube of at least 0.1 mL.

2.   Measure the fluorescence background of each tube in the Beacon® 2000 Fluorescence Polarization System.

3.   Add identical aliquots of the receptor:labeled ligand mixture to each tube, mix, and allow to incubate until equilibrium is established. Alternatively, the receptor and labeled ligand can be added separately if preincubation of these two components is undesirable.

4.   Measure the anisotropy value of each tube.

The competition curve data, mA vs. unlabeled competitor concentration, can be analyzed by computer-assisted non-linear regression yielding IC_{50} values for each of the competitors.

Kinetic Experiments

Determination of the Dissociation Rate Constant, k_{-1}

The calculation of the dissociation rate constant (k_{-1}) is commonly accomplished by binding a ligand to a receptor and measuring the rate of dissociation of receptor from the ligand. In solid-phase binding experiments, ligand is allowed to bind to immobilized receptor and then the incubation medium is quickly removed from the dish or filter and replaced with buffer. This step immediately removes any free and unbound ligand from the system (hence the term "instantaneous dilution"). As the bound ligand reaches a new equilibrium with the ligand in the buffer (zero, at time = 0), the amount of ligand bound to receptor will decrease over time.

One of the primary advantages of fluorescence polarization experiments is that bound and free molecular species do not need to be separated. In dissociation experiments, however, one must find a way to disrupt the binding equilibrium and encourage dissociation of the ligand from the receptor. One method is to add a large excess of an **unlabeled** ligand to the system so that when a labeled ligand dissociates from its partner it is unlikely that labeled ligand will rebind in the face of an overwhelming concentration of unlabeled ligand. This result effectively produces a situation of infinite dilution in relation to the labeled ligand.

1. Select labeled ligand and unlabeled receptor concentrations that will result in 50% bound ligand (50% of maximal mA value). Choose the lowest ligand concentration possible so that you can still see a signal (refer to **Table 8-2**).

2. Determine the mA_{max} of this system by allowing the mixture to reach equilibrium.

3. After the mixture has reached equilibrium, add 100X the K_d of unlabeled ligand to the tube in a small volume and follow anisotropy values over time.

4. From the mA vs. time curve, estimate the mA_{min} value by curve-fitting analysis or visual inspection.

5. Plot the $\ln[(mA_{obs} - mA_{min})/(mA_{max} - mA_{min})]$ vs. time for each time point. This will yield a straight line or curve, with terminal slope $-k_{-1}$. When $mA_{obs} = 0.5\ mA_{max}$, then $k_{-1} = 0.693/T_{1/2}$, where $T_{1/2}$ is the half-time.

This method for the determination of the dissociation constant will yield reliable results unless the binding exhibits positive or negative cooperativity. In the case of positive cooperativity, the addition of unlabeled ligand will decelerate the dissociation of the labeled ligand, resulting in an underestimation of the dissociation rate. In the case of negative cooperativity, the addition of unlabeled ligand will result in an increased dissociation rate (*e.g.*, with the insulin receptor).

Determination of the Association Rate Constant, k_1

The approximate determination of the association rate constant is dependent on B << R_T. In this case, our rate equations simplify to **Equation 8.13:**

$$\textbf{Equation 8.13:} \qquad \textbf{B}_\textbf{t} = \textbf{B}_\textbf{e} \times \{1 - e^{[-(k_1R_T + k_{-1})t]}\}$$

where B_t is bound at time t, B_e is bound at equilibrium, R_T = [total unlabeled receptor], and t = time. When $B_t = 0.5B_e$, **Equation 8.13** simplifies to **Equation 8.14**:

$$\textbf{Equation 8.14:} \qquad \frac{\textbf{ln(2)}}{\textbf{T}_\textbf{1/2}} = \textbf{k}_\textbf{1}\textbf{R}_\textbf{T} + \textbf{k}_\textbf{-1}$$

where $T_{1/2}$ is the half-time of association.

The rate constants are therefore calculated by determining the $T_{1/2}$ for each curve and plotting $\ln(2)/T_{1/2}$ vs. R_T. The $T_{1/2}$ can be estimated in two ways. First, the curvilinear association curve can be analyzed by computer-assisted non-linear regression and $T_{1/2}$ calculated from the fitted curve. Alternatively, the association curve can be linearly transformed and the value of $T_{1/2}$ can be estimated graphically. This second method is now outlined:

1. Empirically select concentrations of ligand and tracer that will generate a binding association curve that can be easily resolved (*e.g.*, binding that does not reach equilibrium in less than 2 minutes). The Beacon® 2000 Analyzer is used in its kinetic mode and data points are taken every 13 seconds.

2. Repeat the association experiment at a fixed ligand concentration and 5–10 different receptor concentrations.

3. Collect data on anisotropy vs. time, for each experiment.

4. Transform the anisotropy data for each curve by subtracting the zero time anisotropy value from each subsequent value.

5. For each curve, estimate the equilibrium plateau in mA units (mA_{eq}).

6. For each curve, plot $(mA_{eq} - mA_t)/mA_{eq}$ vs. time.

7. Determine the $T_{1/2}$ for each curve as the point at which $\log (mA_{eq} - mA_t)/mA_{eq} = 0.5$.

8. Plot $\ln(2)/T_{1/2}$ vs. total receptor concentration. According to **Equation 7.19** (see **Chapter 7**), this plot will yield a straight line with a slope = k_1 and the y-intercept-= k_{-1}.

CHAPTER 9
Products

CoreHTS Estrogen Receptor–α and –β Competitor Assay Kits

Product	Product No.	Quantity
Estrogen Receptor-alpha (ER-α) Competitor Assay	P2698	500 assays
Estrogen Receptor-alpha (ER-α) Competitor Assay	P2614	2,000 assays
Estrogen Receptor-beta (ER-β) Competitor Assay	P2700	500 assays
Estrogen Receptor-beta (ER-β) Competitor Assay	P2615	2,000 assays

Please inquire for bulk purchases.

Applications

• Drug discovery, endocrine disruptor screening and steroid biochemistry research

• Nonradioactive measurement of estrogen receptor binding capacity by competition

• High-throughput screening

• Measurement of IC_{50} values

• Screening of novel binding compounds

• Identification of environmental estrogens

Description

Estrogen Receptor (ER) Competitor Assays for Estrogen Receptors-alpha and -beta (ER-α and ER-β) are ideal for screening novel estrogen receptor binding compounds and endocrine disruptors. The ER Competitor Assays use purified baculovirus-expressed human ER-α or ER-β and Fluormone™ ES2, a new, proprietary fluorescein-labeled estrogen ligand that provides excellent signal-to-noise ratios.

In the ER Competitor Assays, an ER/ES2 complex with a high polarization value is added to a test compound. If the compound competes for estrogen binding, it will displace the ES2 from the ER/ES2 complex causing a reduction in the polarization value. Noncompetitors will not displace the ES2 from the ER/ES2 complex, and the polarization value will remain high. The shift in polarization in the presence of a test compound is used to determine the relative affinity of the compound for either ER-α or ER-β.

Kit Components

P2698

Fluormone™ ES2	125 μL
ER-α, Human Recombinant	1×750 pmol
ES2 Screening Buffer	1×120 mL

P2614

Fluormone™ ES2	500 μL
ER-α, Human Recombinant	4×750 pmol
ES2 Screening Buffer	2×120 mL

P2700

Fluormone™ ES2	125 μL
ER-β, Human Recombinant	1×750 pmol
ES2 Screening Buffer	1×120 mL

P2615

Fluormone™ ES2	500 μL
ER-β, Human Recombinant	4×750 pmol
ES2 Screening Buffer	2×120 mL

Competitors	IC_{50} (nM)
DES	5.4
4-OH Tamoxifen	29.6
Estradiol	5.7
Tamoxifen	60.9

Competitor	IC_{50} (nM)
Tamoxifen	189
DES	3.5
4-OH Tamoxifen	26
Estradiol	5.6
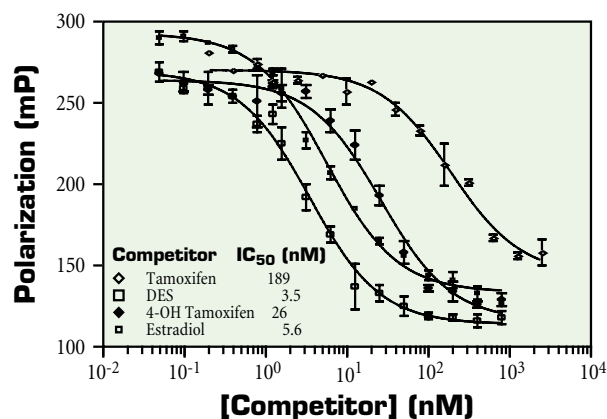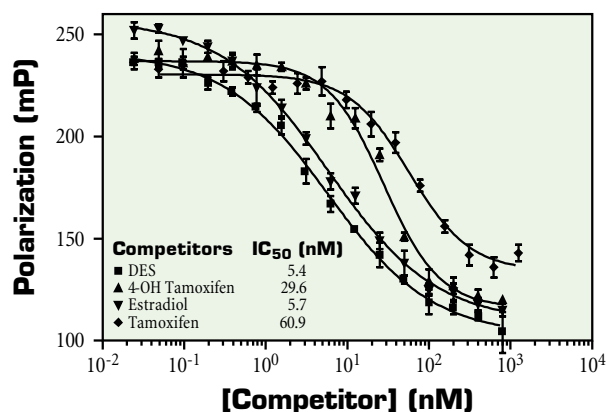
Determination of IC_{50} values by competitive analysis. Test compounds were serially diluted in triplicate in ES2 Screening Buffer. Each dilution series contained 16 wells of diluted compound in 50 μL of ES2 Screening Buffer. A complex of ER-α (left panel) or ER-β (right panel) and ES2 (1 nM) was added to each reaction well for a final volume of 100 μL in each well. Negative controls containing ER and Fluormone™ ES2 (equivalent to 0% inhibition), and positive controls containing only free Fluormone™ ES2 (equivalent to 100% inhibition) were prepared in triplicate. All polarization values were measured after a 2-hour incubation at room temperature. Polarization values (mean ± 1 SD) were plotted against the logarithm of competitor concentration. IC_{50} values (concentration of competitor that displaces half of the Fluormone™ ES2 from ER) were determined from the plot using nonlinear least-squares analysis.

Progesterone Receptor Competitor Assay Kits

Product No.	Quantity
P2895	100 assays
P2896	1,000 assays

Please inquire for bulk purchases.

Applications

- Ideal for drug discovery and steroid biochemistry research
- Nonradioactive measurement of progesterone receptor binding capacity by competition
- Measures true equilibrium binding
- High-throughput screening
- Measurement of IC_{50} values
- Screening of novel binding compounds

Features

- **Ease of Use:** Simple "mix and read" design. Fluorescence polarization-based assay is rapid, homogeneous, and performed in solution
- **Versatile:** Formatted for use with 96-well or 384-well plates
- **Stable:** Assay stable for 3 hours at room temperature

Description

Progesterone Receptor (PR) Competitor Assay Kit, based on fluorescence polarization is ideal for screening novel Progesterone receptor binding compounds. This homogenous assay utilizes an fusion of glutathione transferase to the ligand binding domain of human progesterone receptor [PR-LBD(GST)] and a proprietary fluorescently-tagged progesterone ligand, Fluormone™ PL Green. PanVera®'s new assay provides a rapid and sensitive method for identification of novel PR ligands in a high-throughput format.

PR Competitor Assay

Several compounds were investigated for their ability to compete with PL Green binding to PR–LBD(GST). Competitors were serially diluted in microtiter plates. Purified PR–LBD(GST)/Fluormone™ PL Green complexes were then added to each well to a final concentration of 40 nM PR-LBD(GST) in buffer containing 100 mM potassium phosphate (pH 7.4), 10% glycerol, 100 µg/mL BGG, and 2 mM DTT. Following a one-hour room temperature incubation, the fluorescence polarization value of each well was read and an IC_{50} value was determined for each competitor. Progesterone is the most potent competitor with an IC_{50} value of 25 nM, followed by the anti-progestin RU486 with an IC_{50} of 60 nM. Testosterone and dexamethasone were much weaker competitors with IC_{50} values of 500 nM and 1.4 µM, respectively. PR-LBD(GST)/Fluormone™ PL Green complexes were found to be stable for several hours at room temperature.

Kit Components

P2895

Fluormone™ PL Green	50 µL
PR-LBD(GST)	400 pmol
PR Screening Buffer	20 mL
1 M DTT	1 mL

P2896

Fluormone™ PL Green	500 µL
PR-LBD(GST)	2 × 200 pmol
PR Screening Buffer	200 mL
1 M DTT	1 mL
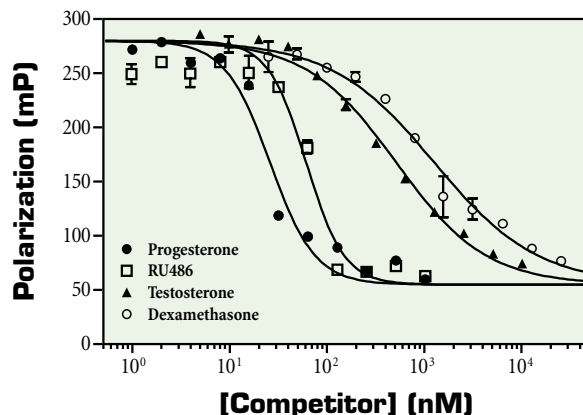
Competition Assay with Fluormone™ PL Green. Steroids were serially diluted in PR Screening Buffer in microtiter plates. PR-LBD(GST)/Fluormone™ PL Green complexes were added to each well to a final concentration of 40 nM and 2 nM, respectively. Following a one-hour room temperature incubation, the fluorescence polarization of each well was read, and for each competitor, an IC_{50} value was determined. The polarization readings were found to be stable for at least three hours at room temperature, and >24 hours at 4°C. Z' = 0.69, which indicates a large separation band between positive and negative controls and a superior quality assay (1).

Reference

Zhang *et al.* (1999) *J. Biomol. Screen.* **4:**67-73.

CoreHTS® Glucocorticoid Receptor Competitor Assay Kit, Green

Product No.	Quantity
P2816	100 assays

Please inquire for bulk purchases.

Applications

- Ideal for drug discovery and steroid biochemistry research
- Nonradioactive measurement of glucocorticoid receptor binding capacity by competition
- High-throughput screening
- Measurement of IC_{50} values
- Screening of novel binding compounds
- Measures true equilibrium binding

Features

- **Ease of Use:** Simple "mix and read" design. Fluorescence polarization-based assay is rapid, homogeneous, and performed in solution
- **Versatile:** Formatted for use with 96-, 384- and 1536-well plates
- **Stable:** Assay stable for 3 hours at room temperature

Description

Glucocorticoid Receptor (GR) Competitor Assay Kit, Green, which is based on fluorescence polarization is ideal for screening novel glucocorticoid receptor binding compounds. The GR Competitor Assay uses partially purified baculovirus-expressed GR and Fluormone™ GS1, a proprietary fluorescein-labeled glucocorticoid ligand. In the GR Competitor Assay, GR is added to the fluorescent ligand in the presence of test compounds in microwells. Test compounds that compete for binding to GR prevent the formation of a ligand/GR complex with high polarization. The shift in polarization in the presence of test compounds is used to determine the relative affinity of test compounds for GR.

The GR Competitor Assay Kit, Green, is designed for room temperature assays, using a coactivator-related Stabilizing Peptide to maintain GR complex integrity (1). The assay can also be run in the absence of the Stabilizing Peptide at 4˚C. The Z' Factor was determined to be 0.56.

Kit Components

Fluormone™ GS1	50 μL
Glucocorticoid Receptor (GR), rHuman	2×25 pmol
GR Screening Buffer, 10X	2×1 mL
GR Stabilizing Peptide, 10X	2×1 mL
DTT, 1 M	1 mL

Competitor	IC_{50} (nM)
■ Dexamethasone	11.4
○ Cortisol	30.5
△ Testosterone	1230
▽ Estradiol	1910

Glucocorticoid competition with Fluormone™ GS1. Serial dilutions of steroids were prepared. GS1 and GR were added sequentially to each well. Final incubation conditions were 4 nM GR, 1 nM GS1, 10 mM potassium phosphate (pH 7.4), 20 mM Na_2MoO_4, 0.1 mM EDTA, 5 mM DTT, 100 μM stabilizing peptide, and 2% DMSO. After a 1-hour incubation at room temperature, the fluorescence polarization of each well was read, using wells containing 4 nM GR as blanks. An IC_{50} and Relative Binding Affinity (RBA) for each steroid was determined from the average of 10 plate reads. Note that the RBA's reflect literature values. (2, 3) Competition experiments were repeated in the absence of Stabilizing Peptide, incubating plates 4 hours at 4˚C. A table of IC_{50} values obtained with various steroids is shown below.

	Room Temperature		4˚C (no peptide)	
Ligand	IC_{50} (nM)	RBA	IC_{50} (nM)	RBA
Dexamethasone	11.4	100	9.9	100
Cortisol	30.5	38	35.7	28
Testosterone	1230	0.93	2710	0.37
β-Estradiol	1910	0.60	6810	0.15
Beclomethasone monoproprionate	6.9	167	4.7	211
Fluocinonide	40.7	28	50.7	19
Prednisone	670	1.71	1560	0.63

References

1. Chang, C.Y. *et. al.* (1999) *Mol. Cell. Biol* **19**:8226-39.
2. Mulatero, P. *et al.* (1997) *Hypertension* **30**:1274-8.
3. Hogger, P. and Rohdewald, P. (1994) *Steroids* **59**:597-602.

Bibliography

- Beato, M. *et. al.* (1995) *Cell* **83**:851-7.
- Srinivasan, G. *et al.* (1990) *Mol. Endo.* **4**:209-16.
- Tsai, M. *et al.* (1994) *Annu. Rev. Biochem.* **63**:451-86.
- Vottero, A. *et al.* (1999) *TEM* **10**:333-8.

CoreHTS® Glucocorticoid Receptor Competitor Assay Kit, Red

Product No.	Quantity
P2893	**100 assays**

Please inquire for bulk purchases.

Applications

- Ideal for drug discovery and steroid biochemistry research
- Nonradioactive measurement of glucocorticoid receptor binding capacity by competition
- High-throughput screening
- Measurement of IC_{50} values
- Screening of novel binding compounds
- Measures true equilibrium binding

Features

- **Red Emission:** Avoids intrinsic fluorescence of compounds sometimes found in the "green" region of the visible spectrum
- **Ease of Use:** Simple "mix and read" design. Fluorescence polarization-based assay is rapid, homogeneous, and performed in solution
- **Versatile:** Formatted for use with 96-, 384- and 1536-well plates
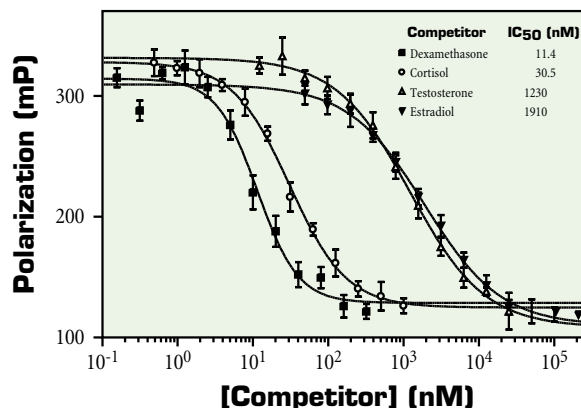- **Stable:** Assay stable for 3 hours at room temperature

Description

Glucocorticoid Receptor (GR) Competitor Assay Kit, Red, which is based on fluorescence polarization, is ideal for screening novel glucocorticoid receptor binding compounds. The GR Competitor Assay, Red, uses partially purified baculovirus-expressed GR and Fluormone™ GS Red, a new, proprietary fluorescent glucocorticoid ligand that eliminates the intrinsic fluorescence found in some compounds when excited in the green region of the visible spectrum.

In the GR Competitor Assay, Red, GR is added to the fluorescent ligand in the presence of test compounds in microwells. Test compounds that compete for binding to GR prevent the formation of a ligand/GR complex with high polarization. The shift in polarization in the presence of test compounds is used to determine the relative affinity of test compounds for GR.

The GR Competitor Assay Kit, Red, contains reagents is designed for room temperature assays, using a coactivator-related Stabilizing Peptide to maintain GR complex integrity (1). The assay can also be run in the absence of the Stabilizing Peptide at 4˚C. The Z' Factors were determined to be 0.85 (data not shown).

Kit Components

Fluormone™ GS Red	50 μL
Glucocorticoid Receptor (GR), rHuman	2×25 pmol
GR Screening Buffer, 10X	2×1 mL
GR Stabilizing Peptide, 10X	2×1 mL
DTT, 1 M	1 mL
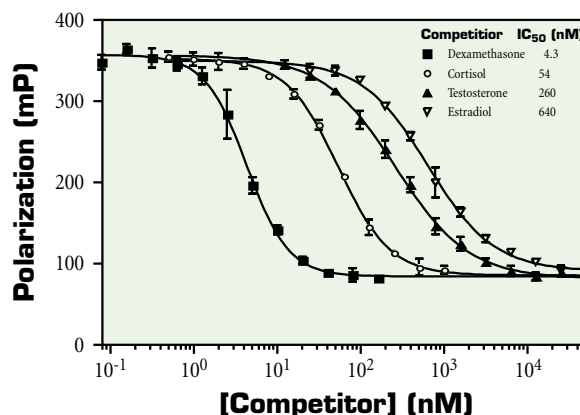
Steroid competition with Fluormone™ GS Red. Serial dilutions of steroids were prepared. GS Red and GR were added sequentially to each well. Final incubation conditions were 4 nM GR, 1 nM GS Red, 10 mM potassium phosphate (pH 7.4), 20 mM Na_2MoO_4, 0.1 mM EDTA, 5 mM DTT, 100 μM stabilizing peptide, and 2% DMSO. After a 2-hour incubation at room temperature, the fluorescence polarization of each well was read, using wells containing 4 nM GR as blanks. An IC_{50} for each steroid was determined from the average of 10 plate reads.

Bibliography

- Beato, M. *et. al.* (1995) *Cell* **83**:851-7.
- Srinivasan, G. *et al.* (1990) *Mol. Endo.* **4**:209-16.
- Tsai, M. *et al.* (1994) *Annu. Rev. Biochem.* **63**:451-86.
- Vottero, A. *et al.* (1999) *TEM* **10**:333-8.

Tyrosine Kinase Assay Kits, Green and Red

Product	Product No.	Quantity
Tyrosine Kinase Assay Kit, Green	P2836	100 assays
Tyrosine Kinase Assay Kit, Green	P2837	1,000 assays
Tyrosine Kinase Assay Kit, Red	P2882	100 assays
Tyrosine Kinase Assay Kit, Red	P2883	1,000 assays

Please inquire for bulk purchases.

Applications

- Signal transduction research and drug discovery
- Nonradioactive detection of tyrosine kinases
- High-throughput screening
- Quantitative measurement of inhibitor IC_{50} values
- Screening of novel tyrosine kinase inhibitors

Features

- **Red Assay Kit:** Red emission avoids intrinsic fluorescence found in the "green" wavelength-region of the visible spectrum
- **Improved Sensitivity**
- **Fluorescence polarization-based:** Assay is rapid, homogeneous, and performed in solution.
- **Versatile:** Formatted for use with single tubes, or multiwell plates.
- **Flexible:** Antibody and tracer are supplied as separate components.
- **Stable:** Measurements can be made for up to 48 hours.

Description

Protein tyrosine kinases (PTKs) play a role in many cellular processes, including differentiation, growth, metabolism, and apoptosis. PanVera®'s Tyrosine Kinase Assay Kits, Red, utilize a red-shifted fluorescently-labeled phosphopeptide tracer that has the added benefit of minimizing background interference occasionally found in compound libraries. The Green and Red Assay Kits contain the materials necessary to analyze the products of a protein tyrosine kinase reaction using fluorescence polarization as the detection method. The principle behind this assay is competition: a fluorescent phosphopeptide tracer and the non-fluorescent phosphopeptides generated during a tyrosine kinase assay compete for binding to an antiphosphotyrosine antibody. In a reaction mixture containing no phosphopeptide product, the fluorescent tracer is bound by the antibody and the emission signal is polarized; however, in a reaction mixture containing phosphopeptide product, the tracer is displaced from the antibody and the emission signal becomes depolarized. Tyrosine Kinase Assay Kits are available in 100 and 1,000 assay sizes. The anti-phosphotyrosine antibody and tracer are now supplied separately for more flexibility.

Kit Components

P2836
Anti-Phosphotyrosine Antibody,10X	1 mL
PTK Green Tracer,10X	1 mL
PTK Competitor	200 µL
PTK Quench Buffer	1 mL
FP Dilution Buffer	5 mL

P2837
Anti-Phosphotyrosine Antibody,10X	10 mL
PTK Green Tracer,10X	10 mL
PTK Competitor	200 µL
PTK Quench Buffer	10 mL
FP Dilution Buffer	50 mL

P2882
Anti-Phosphotyrosine Antibody, 10X	1 mL
PTK Red Tracer, 10X	1 mL
PTK Competitor	200 µL
PTK Quench Buffer	1 mL
FP Dilution Buffer	5 mL

P2883
Anti-Phosphotyrosine Antibody, 10X	10 mL
PTK Red Tracer, 10X	10 mL
PTK Competitor	200 µL
PTK Quench Buffer	10 mL
FP Dilution Buffer	50 mL
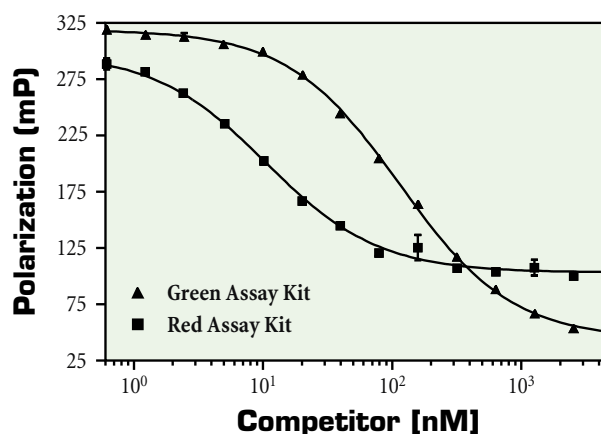
Competition Curve Using Phosphotyrosine Peptide in PanVera®'s Tyrosine Kinase Assay Kits,Green and Red. The IC_{50} for the TK Competitor was 11 nM in the Red Kit and 115 nM in the Green Kit (n = 4). A 2-fold serial dilution of the competitor was performed in a 50 µL volume of FP Dilution Buffer. 10 µL of 10X Anti-phosphotyrosine Antibody and 10 µL of 10X TK Tracer, either Green or Red, were added to 30 µL of FP Dilution Buffer for a final volume of 100 µL. The samples were protected from light and incubated at room temperature for 1 hour, then read on the TECAN Ultra in a 96-well black, round bottom DYNEX plate.

Peptide substrates for tyrosine kinases. The binding to phosphotyrosines in this kit is sequence-independent. To demonstrate this, at concentrations relevant to an in vitro kinase reaction, the following tyrosine-phosphorylated peptides have been shown to completely displace the F-phosphopeptide tracer from the anti-phosphotyrosine antibody included in this kit:

Peptide Substrate Sequence	Enzymes That Utilize These Substrates
poly(Glu,Tyr)4:1	Zap70, EGF Receptor, pp60 c-Src, Src N1, Src N2, Lck
ADEYLIPQQ	EGF Receptor
DEDYIQD	EGF Receptor
KVEKIGEGTYGVV	Fyn, Lck, Lyn, pp60 c-Src
RRDIYETD	Insulin Receptor
EYLIPQ	EGF Receptor
EILYNMDEG	EGF Receptor
KQVVDDSATYNVIK	EGF Receptor
QEEYSAM	H-ras 1/p21
TAENAEYLRVAPQ	EGF Receptor
SLDNPDYQQDFFP	EGF Receptor
SVQNPVYHNQPLN	EGF Receptor
FLPVPEYINQSVP	EGF Receptor
HTDDEMTGYVATR	MKK6

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Ser/Thr Kinase Assay Kits, Crosstide

Product	Product No.	Quantity
Ser/Thr Kinase Assay Kit, Crosstide	P2886	100 assays
Ser/Thr Kinase Assay Kit, Crosstide	P2887	1,000 assays
Substrate, Crosstide Assay, 10 μM	P2926	1 mL

Please inquire for bulk purchases.

Applications

- Ideal for signal transduction research and drug discovery
- Nonradioactive detection of Serine/Threonine kinases
- High-throughput screening
- Quantitative measurement of inhibitor IC_{50} values
- Screening of novel Serine/Threonine inhibitors

Features

- **Fluorescence polarization-based:** Assay is rapid, homogeneous, and performed in solution.
- **Reliable:** Z' Factor of 0.7.
- **Versatile:** Formatted for use with single tubes, or multiwell plates.
- **Flexible:** Antibody and tracer are supplied as separate components.
- **Stable:** Measurements can be made for up to 48 hours.

Description

Kinase phosphorylation of proteins is critical to the normal regulation of many biological mechanisms, including cell growth, apoptosis, and differentiation (1). Serine/threonine kinases can be grouped in part by their ability to recognize sequence motifs within protein substrates. *In vitro* assays using peptide substrates based on a particular motif can be used to measure the activity of multiple kinases. Crosstide, a synthetic peptide corresponding to the sequence in Glycogen Synthase Kinase-3 (GSK3) that contains a serine (9 on GSK 3α and 21 on GSK 3β) which is phosphorylated by many serine/threonine kinases, including, but not limited to, PKB/Akt, MAPKAP-1, RSK, and p70 S6 kinase (2). PanVera®'s assay utilizes the versatility of the Crosstide substrate to allow the researcher to study many kinases in one format. The assay is simple, sensitive, non-radioactive, homogeneous and formatted for high-throughput screening. This assay kit contains all the materials necessary to analyze kinase activity via Crosstide phosphorylation using fluorescence polarization (FP) as the detection method. The kits contain reagent volumes sufficient for one hundred (PanVera® Part No. P2886) and one thousand (PanVera® Part No. P2887) 100 μL assays, respectively. These kits are formatted for use with either a multiwell plate instrument capable of measuring FP in 96-well plates or a single-well FP instrument, such as the Beacon® 2000 System (PanVera® Corporation). These kits also work in 384- and 1536-well plates using reduced reagent volumes.

Kit Components

P2884

Ab, 4X, Crosstide Assay	2.5 mL
Tracer, 10X, Crosstide Assay	1 mL
Competitor, Crosstide Assay, 5 μM	500 μL
FP Dilution Buffer	5 mL
Kinase Quench Buffer	1 mL

P2885

Ab, 4X, Crosstide Assay	25 mL
Tracer, 10X, Crosstide Assay	10 mL
Competitor, Crosstide Assay, 5 μM	500 μL
FP Dilution Buffer	50 mL
Kinase Quench Buffer	10 mL
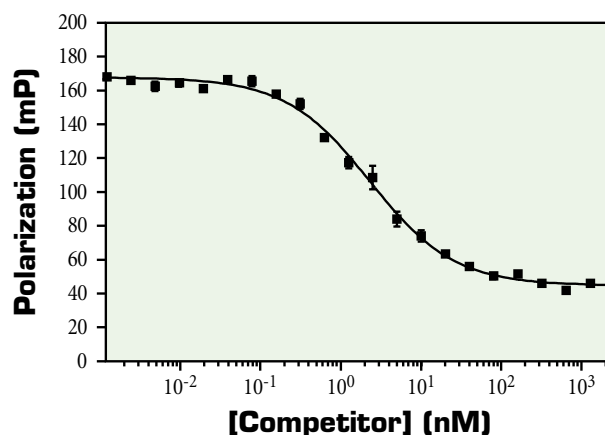
Competition Curve Using PanVera®'s Ser/Thr Kinase Assay Kit, Crosstide. The Crosstide Assay competitor was serially diluted in FP dilution Buffer (from 2.5 μM to 4.8 pM) over 18 wells of a black, 96-well round bottom DYNEX plate, with a post-dilution volume of 50 μL per well. 25 μL of 4X Phosphoserine peptide-specific Antibody, 10 μL of 10X Tracer and 15 μL of FP Dilution Buffer were added to each well, bringing the final volume to 100 μL per well. The plates were protected from light and incubated at room temperature for 1 hour, then read on a TECAN Ultra FP plate reader. The IC_{50} for the competitor was 2 nM (n = 4). The Z' Factor for this assay is 0.7 and the Signal/Noise and Signal/Background readings are 18.6 and 4.1 respectively.

References

1. Hunter, T. (1995) *Cell* **80:**225-36.

2. Cross D.A. *et al.* (1995) *Nature* **378:**785-9.

Protein Kinase C Assay Kits, Green

Product	Product No.	Quantity
Protein Kinase C Assay Kit, Green	P2747	100 assays
Protein Kinase C Assay Kit, Green	P2748	1,000 assays
Protein Kinase C Substrate, 100 µM	P2760	1 mL

Please inquire for bulk purchases.

Applications
- Ideal for signal transduction research and drug discovery
- Nonradioactive detection of PKC isoforms, PKA, and GSK 3β
- High-throughput screening
- Quantitative measurement of inhibitor IC_{50} values
- Screening of inhibitors of PKC isoforms, PKA, and GSK 3β

Features
- **Fluorescence polarization-based:** Assay is rapid, homogeneous, and performed in solution.
- **Versatile:** Formatted for use with single tubes, or multiwell plates.
- **Flexible:** Antibody and tracer are supplied as separate components.
- **Stable:** Measurements can be made for up to 48 hours.

Description

The phosphorylation of serine and threonine residues in proteins by Protein Kinase C (PKC) family members is critical to the normal regulation of many biological mechanisms, including the modulation of membrane structure and cytoskeletal reorganization, receptor desensitization, transcriptional control, cell growth and differentiation, and mediation of immune response. PKCs also play a role in memory, learning, and long-term potentiation. The PKCs influence cellular events via their activation by second messenger pathways that involve the production of diacylglycerol (1, 2). The in vivo regulation of PKC family members involves a combination of the subcellular location of the enzyme(s) and their substrate(s). Identification of specific functions of the different isoforms is dependent on the development of isoform-specific inhibitors (3, 4).

The substrate used in this kit contains the R-X-X-S/T consensus motif, which is recognized by a number of serine/threonine kinases. The activity of PKA, GSK3b, and PKB has been detected using this assay. Other serine/threonine kinases whose substrates contain a similar serine or threonine consensus motif or the motif R-X-S/T may also have measurable activity in this assay.

PanVera®'s Protein Kinase C Assay Kits contain the materials necessary to analyze the products of a PKC reaction using fluorescence polarization as the detection method. The principle behind this assay is competition: a fluorescent phosphopeptide tracer and the nonfluorescent phosphopeptides generated during a PKC reaction compete for binding to an antiphosphoserine peptide-specific antibody. In a reaction mixture containing no phosphopeptide product, the fluorescent tracer is bound to the antibody and the emission signal is polarized. However, in a reaction mixture containing phosphopeptide product, the fluorescent tracer is displaced from the antibody and the emission signal becomes depolarized.

The following PKC isoforms, available from PanVera®, have been successfully tested with this kit: PKCα (alpha), PKCβI (beta I), PKCβII (beta II), PKCγ (gamma), PKCδ (delta), PKCε (epsilon), PKCζ (zeta), PKCη (eta) and PKCθ (theta).

Kit Components

P2747

Anti-Phosphoserine Antibody, 4X	2.5 mL
PKC Tracer, 50X	200 µL
PKC Competitor	100 µL
PKC Standard Curve Dilution Buffer	8 mL
PKC Quench Buffer, 4X	2.5 mL

P2748

Anti-Phosphoserine Antibody, 4X	25 mL
PKC Tracer, 50X	2 mL
PKC Competitor	100 µL
PKC Standard Curve Dilution Buffer	8 mL
PKC Quench Buffer, 4X	25 mL

Activity of PKC isoforms. The indicated PKC isoforms were serially diluted in a round-bottom, black 96-well plate. After a 5-minute incubation with additional PKC reaction components, ATP was added to start the reactions. After a 90-minute incubation, a Quench/Detection mixture (containing EDTA and antiphosphoserine antibodies) was added to each well to stop the reaction and initiate the competition for antibody binding. Following an additional 30-minute incubation, the fluorescence polarization of each well was measured on a TECAN Polarion fluorescence polarization instrument.
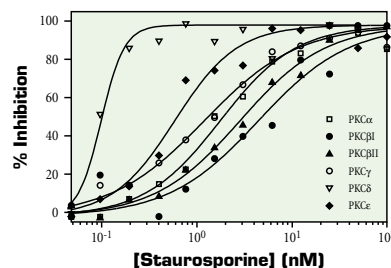
Determination of IC_{50} values for a PKC inhibitor. The indicated PKC isoforms were individually incubated with serial dilutions of Staurosporine (Calbiochem), a common PKC inhibitor, for 5 minutes in a round-bottom, black 96-well plate. ATP was added to start the reactions. After a 90-minute incubation, a Quench/Detection mixture (containing EDTA and antiphosphoserine antibodies) was added to each well to stop the reaction and initiate the competition for antibody binding. Results were measured on a TECAN Polarion fluorescence polarization instrument, and nonlinear regression analysis was performed on a semi-logarithmic plot of the data.

References
1. Nishizuka, Y. (1992) *Science* **258:**607-14.
2. Nishizuka, Y. (1986) *Science* **233:**305-12.
3. Wilkinson, S.E. and Hallam, T.J. (1994) *Trends Pharmacol. Sci.* **15:**53-7.
4. Conrad, R. *et al.* (1994) *J. Biol. Chem.* **269:**32051-4.

CoreHTS Protein Kinase C Assay Kits, Red

Product	Product No.	Quantity
Protein Kinase C Assay Kit, Red	P2940	100 assays
Protein Kinase C Assay Kit, Red	P2941	1,000 assays
Protein Kinase C Substrate, 100 μM	P2760	1 mL

Please inquire for bulk purchases.

Applications

- Ideal for signal transduction research and drug discovery
- Nonradioactive detection of PKC isoforms, PKA, and GSK 3β
- High-throughput screening
- Quantitative measurement of inhibitor IC_{50} values
- Screening of inhibitors of PKC isoforms, PKA, and GSK 3β

Features

- **Red Emission:** Avoids intrinsic fluorescence found in the "green" wavelength-region of the visible spectrum
- **Fluorescence polarization-based:** Assay is rapid, homogeneous, and performed in solution.
- **Versatile:** Formatted for use with single tubes, or multiwell plates.
- **Flexible:** Antibody and tracer are supplied as separate components.
- **Stable:** Measurements can be made for up to 48 hours.

Description

The phosphorylation of serine and threonine residues in proteins by Protein Kinase C (PKC) family members is critical to the normal regulation of many biological mechanisms, including the modulation of membrane structure and cytoskeletal reorganization, receptor desensitization, transcriptional control, cell growth and differentiation, and mediation of immune response. PKCs also play a role in memory, learning, and long-term potentiation. The PKCs influence cellular events via their activation by second messenger pathways that involve the production of diacylglycerol (1, 2). The in vivo regulation of PKC family members involves a combination of the subcellular location of the enzyme(s) and their substrate(s). Identification of specific functions of the different isoforms is dependent on the development of isoform-specific inhibitors (3, 4).

The substrate used in this kit contains the R-X-X-S/T consensus motif, which is recognized by a number of serine/threonine kinases. The activity of PKA, GSK3b, and PKB has been detected using this assay. Other serine/threonine kinases whose substrates contain a similar serine or threonine consensus motif or the motif R-X-S/T may also have measurable activity in this assay.

PanVera®'s Protein Kinase C Assay Kits contain the materials necessary to analyze the products of a PKC reaction using fluorescence polarization as the detection method. The principle behind this assay is competition: a fluorescent phosphopeptide tracer and the nonfluorescent phosphopeptides generated during a PKC reaction compete for binding to an antiphosphoserine peptide-specific antibody. In a reaction mixture containing no phosphopeptide product, the fluorescent tracer is bound to the antibody and the emission signal is polarized. However, in a reaction mixture containing phosphopeptide product, the fluorescent tracer is displaced from the antibody and the emission signal becomes depolarized.

The following PKC isoforms, available from PanVera®, have been successfully tested with this kit: PKCα (alpha), PKCβI (beta I), PKCβII (beta II), PKCγ (gamma), PKCδ (delta), PKCε (epsilon), PKCζ (zeta), PKCη (eta) and PKCθ (theta).
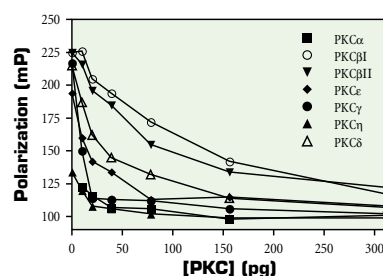
Kit Components

P2940

|	
Ab, 4X, PKC Red Assay	2.5 mL
PKC Red Tracer, 50X	1 mL
PKC Competitor, 10 μM	100 μL
PKC Standard Curve Dilution Buffer	5 mL
Kinase Quench Buffer	2.5 mL

P2941

Ab, 4X, PKC Red Assay	25 mL
PKC Red Tracer, 50X	10 mL
PKC Competitor, 10 μM	100 μL
PKC Standard Curve Dilution Buffer	50 mL
Kinase Quench Buffer	25 mL
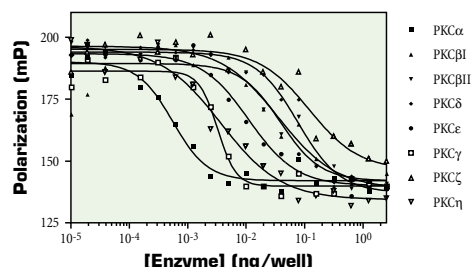
PKC Enzyme Panel Titration Using PanVera®'s PKC Assay Kit, Red. A 2-fold serial dilution of enzyme from 50 ng to 0.1 pg was performed. All reaction components were added to the diluted enzyme on the plate. Final reaction conditions were: 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.02 μM sodium vanadate, 5 μM ATP, 0.1 mg/mL phosphatidylserine, 0.02 mg/mL diacylglycerol, 1X PKC Red Tracer, 4 mM Hepes, 0.02% NP-40, 100 nM PKC Substrate. After a 90 minute incubation at room temperature, we added 25 μL of a 2X PKC Quench/Detection Mix containing equal volumes of PKC Antibody Red, 4X and PKC Quench Buffer, 4X for a final volume of 50 μL in the wells. The samples were read on a TECAN Ultra in a 384-well black, round bottom Labsystems Cliniplate after a 24 hour incubation.

Inhibition Curve with PKC Enzyme Panel Using PanVera®'s PKC Assay Kit, Red. A 2-fold serial dilution of Staurosporine from 4000 ng to 0.122 ng was performed. All reaction components were added to the diluted enzyme on the plate. Final reaction conditions were: 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.02 μM sodium vanadate, 5 ng PKC enzyme, 5 μM ATP, 0.1 mg/mL phosphatidylserine, 0.02 mg/mL diacylglycerol, 1X PKC Red Tracer, 4 mM Hepes, 0.02% NP-40, 100 nM PKC Substrate. After a 90 minute incubation at room temperature, we added 25 μL of a 2X PKC Quench/Detection Mix containing equal volumes of PKC Antibody Red, 4X and PKC Quench Buffer, 4X for a final volume of 50 μL in the wells. The samples were read on a TECAN Ultra in a 384-well black, round bottom Labsystems Cliniplate after a 24 hour incubation.

References

1. Nishizuka, Y. (1992) *Science* **258:**607-14.
2. Nishizuka, Y. (1986) *Science* **233:**305-12.
3. Wilkinson, S.E. and Hallam, T.J. (1994) *Trends Pharmacol. Sci.* **15:**53-7.
4. Conrad, R. *et al.* (1994) *J. Biol. Chem.* **269:**32051-4.

Serine Kinase Assay Kits, IκB-α pSer 32 and pSer 36

Product	Product No.	Quantity
Serine Kinase Assay Kit, IκB-α pSer 32	P2827	100 assays
Serine Kinase Assay Kit, IκB-α pSer 32	P2828	1,000 assay
Serine Kinase Assay Kit, IκB-α pSer 36	P2849	100 assays
Serine Kinase Assay Kit, IκB-α pSer 36	P2850	1,000 assays
IκB-α Peptide, 100 μM, non-phosphorylated	P2826	1 mL

Please inquire for bulk purchases.

Applications

- High-throughput screening of Protein Kinase inhibitors
- Quantitative measurement of inhibitor IC_{50} values

Features

- **Ease of Use:** Simple "mix and read" design. Fluorescence polarization-based assay is rapid, homogeneous, and performed in solution.
- **Versatile:** Formatted for use with either single tubes or microwell plates.

Description

Phosphorylation of IκB-α at serines 32 and 36 results in the activation and nuclear translocation of NF-κB. This phosphorylation is activated by a diverse group of extracellular signals, including virus infection, and oxidative stress, and is a key regulatory point in the initiation of immune or inflammatory responses.

PanVera®'s Serine Kinase Assay Kits, IκB-α pSer 32 and pSer36 contains the materials necessary to quantitate the products of a kinase reaction in which the phosphorylated substrate contains the amino acid sequences surrounding phosphoserine 32 or 36 of IκB-α. The detection method used is fluorescence polarization. The principle behind these assays is competition: a fluorescent phosphopeptide tracer and the nonfluorescent phosphopeptides generated during a protein kinase reaction compete for binding to an antibody specific for either the phosphorylated Serine 32 or Serine 36 on the phosphorylated IκB-α peptide. In a reaction mixture containing no phosphopeptide product, the fluorescent tracer is bound by the antibody and the emission signal is polarized. However, in a reaction mixture containing phosphopeptide product, the fluorescent tracer is displaced from the antibody and the emission signal becomes depolarized. The decrease in polarization values correlates to enzyme activity/inhibition. These assays are optimized for HTS in 384- and 1536-well plates.

Kit Components

P2827

Anti-IκB-α pSer 32 Antibody, 10X	1 mL
IκB-α pSer 32 Tracer, 10X	1 mL
IκB-α pSer 32 Competitor	100 μL
IκB-α pSer 32 Dilution Buffer	4 mL
Kinase Quench Buffer	1 mL

P2828

Anti-IκB-α pSer 32 Antibody, 10X	10 mL
IκB-α pSer 32 Tracer, 10X	10 mL
IκB-α pSer 32 Competitor	100 μL
IκB-α pSer 32 Dilution Buffer	40 mL
Kinase Quench Buffer	10 mL

Kit Components continued...

P2849

Anti-IκB-α pSer 36 Antibody, 10X	1 mL
IκB-α pSer 36 Tracer, 10X	1 mL
IκB-α pSer 36 Competitor	500 μL
FP Dilution Buffer	5 mL
Kinase Quench Buffer	1 mL

P2850

Anti-IκB-α pSer 36 Antibody, 10X	10 mL
IκB-α pSer 36 Tracer, 10X	10 mL
IκB-α pSer 36 Competitor	500 μL
FP Dilution Buffer	50 mL
Kinase Quench Buffer	10 mL
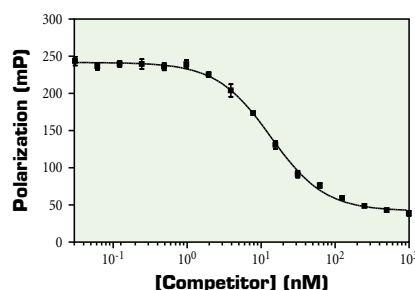
Competition Curve Using PanVera®'s Serine Kinase Assay Kit, IκB-α pSer 32. A 2-fold serial dilution of the competitor from 1000 nM to 0.030 nM was performed in IκB-α pSer 32 Dilution Buffer. Ten microliters of Anti-IκB-α pSer 32 Antibody, 10X and 10 μL of IκB-α pSer 32 Tracer, 10X were added to the 80 μL diluent for a final volume of 100 μL. The samples were protected from light and incubated at room temperature for 2 hours, then read on the TECAN Polarion in a 96-well black, round bottom DYNEX plate. The IC_{50} for the IκB-α pSer 32 Competitor was 13.5 nM (n = 4).
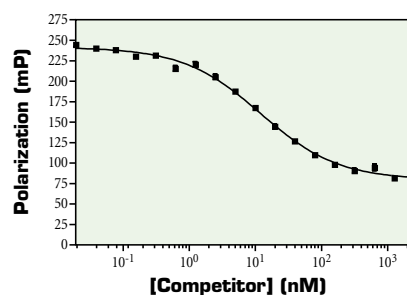
Competition Curve Using PanVera®'s Serine Kinase Assay Kit, IκB-α pSer 36. A 2-fold serial dilution of the competitor from 2.5 μM to 0.02 nM was performed in a 50 μL volume of FP Dilution Buffer in a black 96-well, round bottom Dynex plate. 10 μL of Anti-IκB-α pSer 36 Antibody, 10X and 10 μL of IκB-α pSer 36 Tracer, 10X and 30 μL of FP Dilution Buffer were added to each well for a final volume of 100 μL. The samples were protected from light and incubated at room temperature for 1 hour, then read on a TECAN Polarion FP plate reader (Research Triangle Park, NC). The IC_{50} for the IκB-α pSer 36 competitor was 12 nM (n = 4).

CoreHTS® CDK Assay Kits, Rb[ING]

Product	Product No.	Quantity
CDK Assay Kit, Rb[ING]	P2928	100 assays
CDK Assay Kit, Rb[ING]	P2929	1,000 assays
RB[ING] Substrate, 10 µM	P2939	1 mL

Please inquire for bulk purchases.

Applications
- Ideal for signal transduction research and drug discovery
- Nonradioactive detection of Serine/Threonine kinases
- High-throughput screening
- Quantitative measurement of inhibitor IC_{50} values
- Screening of novel cyclin-dependent kinase inhibitors

Features
- **Fluorescence polarization-based:** Assay is rapid, homogeneous, and performed in solution.
- **Reliable:** Z' Factor of 0.7.
- **Versatile:** Formatted for use with single tubes, or multiwell plates.
- **Flexible:** Antibody and tracer are supplied as separate components.
- **Stable:** Measurements can be made for up to 48 hours.

Description
The retinoblastoma protein (Rb) is known as a tumor suppressor protein that acts via inhibition of cell cycle progression from the G_1 to the S phase. Evidence also supports a more general function of Rb at the transcriptional and cellular levels (1). In addition to its involvement in not only regulating an elusive cell cycle checkpoint, Rb may also have roles in oncogenic viral infection, cellular differentiation and apoptosis. Rb's control over cell cycle progression is determined by its phosphorylation by Cyclin Dependant Kinases (CDKs). One site on Rb that is phosphorylated and has an important impact on the regulation of cellular events is Threonine 252 (2, 3). Because of CDK and Rb's involvement in many important cell-regulatory processes, they are potentially important targets for therapeutic drug discovery research. Conventional kinase assays are tedious, utilize radioactive reagents, and are not easily automated or converted to a high-throughput format for drug screening. PanVera®'s CDK Assay Kit, Rb[ING] is a major advance because it is simple, sensitive, non-radioactive, homogeneous and formatted for high-throughput screening. This assay kit contains all the materials, except the kinase, necessary to analyze CDK-like kinase activity via phosphorylation of Rb at Thr252 (Rb[ING]) using fluorescence polarization (FP) as the detection method. The CDK Assay Kits, Rb[ING] contain reagent volumes sufficient for one hundred (PanVera® Part No. P2928) and one thousand (PanVera® Part No. P2929) 100 µL assays, respectively. These kits are formatted for use with either a multi-well plate instrument capable of measuring FP in 96-well plates or a single-well FP instrument, such as the Beacon® 2000 System (PanVera® Corporation). These kits also work in 384- and 1536-well plates using reduced reagent volumes.

Kit Components

P2928
Ab, 4X, CDK Assay, Rb[ING]	2.5 mL
Rb[ING] Tracer, 10X	1 mL
Rb[ING] Competitor, 5 µM	500 µL
FP Dilution Buffer	5 mL
Kinase Quench Buffer	1 mL

P2929
Ab, 4X, CDK Assay, Rb[ING]	25 mL
Rb[ING] Tracer, 10X	10 mL
Rb[ING] Competitor, 5 µM	500 µL
FP Dilution Buffer	50 mL
Kinase Quench Buffer	10 mL
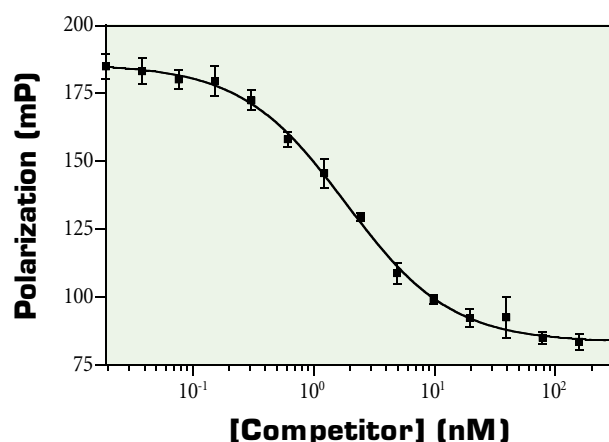
Competition Curve Using PanVera®'s Cyclin Dependent Kinase Assay Kit, Rb[ING]. The Rb[ING] competitor was serially diluted in FP dilution Buffer (from 2.5 µM to 4.8 pM) over 18 wells of a black, 96-well round bottom DYNEX plate, with a post-dilution volume of 50 µL per well. 25 µL of 4X Anti-Rb[ING] Antibody, 10 µL of 10X Rb[ING] Tracer and 15 µL of FP Dilution Buffer were added to each well, bringing the final volume to 100 µL per well. The plates were protected from light and incubated at room temperature for 1 hour, then read on a TECAN Ultra Fluorescence Polarization plate reader. The IC_{50} for the Rb[ING] competitor was 2 nM (n = 4). The Z' Factor for this assay is 0.7.

References
1. Kaelin, W.G. (1999) *BioEssays* **21:**950-8.
2. Konstantinidis, A.K. *et al.* (1998) *J. Biol. Chem.* **273:**26506-15.
3. Zarkowska, T. and Mittnacht, S. (1997) *J. Biol. Chem.* **272:**12738-46.

CoreHTS® Threonine Kinase Assay Kits, PDK1

Product	Product No.	Quantity
Threonine Kinase Assay Kit	P2884	100 assays
Threonine Kinase Assay Kit	P2885	1,000 assays
PDK1 Substrate Peptide, 100 μM	P2925	1 mL

Please inquire for bulk purchases.

Applications

- Ideal for signal transduction research and drug discovery
- Nonradioactive detection of serine/threonine kinases
- High-throughput screening
- Quantitative measurement of inhibitor IC_{50} values
- Screening of novel serine/threonine kinase inhibitors

Features

- **Fluorescence polarization-based:** Assay is rapid, homogeneous, and performed in solution.
- **Versatile:** Formatted for use with single tubes, or multiwell plates.
- **Flexible:** Antibody and tracer are supplied as separate components.
- **Stable:** Measurements can be made for up to 48 hours.

Description

PDK1 (3-Phosphoinositide-Dependent Protein Kinase-1) activates the conventional PKCs and PKCζ (zeta) through phosphorylation at Threonine 500 in the activation loop. PDK1 also phosphorylates Protein Kinase B (PKB/Akt) at Threonine 308 in the presence of phosphatidylinositol-3,4,5-trisphosphate. Active PKB/Akt inactivates Glycogen Synthase Kinase-3 (GSK 3), eventually leading to the dephosphorylation and activation of glycogen synthase and the stimulation of glycogen synthesis. Because of the role PDK1 plays in insulin-induced glycogen synthesis and PKC activation, it is potentially an important target for metabolic drug research.

Conventional serine/threonine kinase assays are tedious, utilize radioactive reagents, and are not easily automated or converted to a high-throughput format for drug screening. PanVera®'s PDK1 assay kit is a major advance because it is simple, sensitive, non-radioactive, homogeneous and formatted for high-throughput screening. The Threonine Kinase Assay Kit, PDK1 contain reagent volumes sufficient for one hundred (PanVera® Part No. P2884) and one thousand (PanVera® Part No. P2885) 100 μL assays, respectively. These kits are formatted for use with either a multiwell plate instrument capable of measuring FP in 96-well plates or a single-well FP instrument, such as the Beacon® 2000 System (PanVera® Corporation). These kits also work in 384- and 1536-well plates using reduced reagent volumes.

Kit Components

P2884

Anti-Phosphothreonine Antibody, 4X	2.5 mL
PDK1 Tracer, 10X	1 mL
PDK1 Competitor, 5 μM	500 μL
FP Dilution Buffer	5 mL
Kinase Quench Buffer	1 mL

P2885

Anti-Phosphothreonine Antibody, 4X	25 mL
PDK1 Tracer, 10X	10 mL
PDK1 Competitor, 5 μM	500 μL
FP Dilution Buffer	50 mL
Kinase Quench Buffer	10 mL
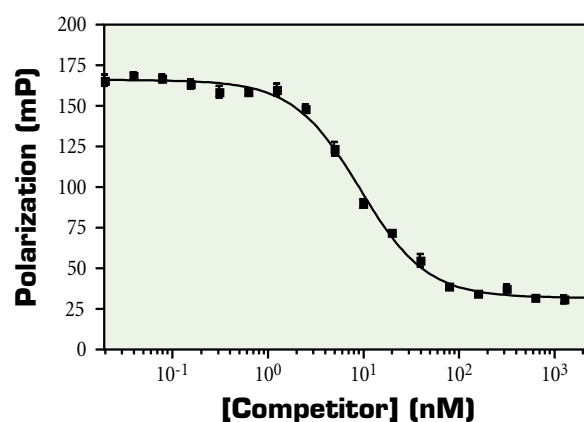
Competition Curve Using PanVera®'s PDK1 Kinase Assay Kit. The PDK1 competitor was serially diluted in FP dilution Buffer (from 2.5 μM to 4.8 pM) over 18 wells of a black, 96-well round bottom DYNEX plate, with a post-dilution volume of 50 μL per well. 25 μL of 4X Anti-PDK1 Antibody, 10 μL of 10X PDK1 Tracer and 15 μL of FP Dilution Buffer were added to each well, bringing the final volume to 100 μL per well. The plates were covered to protect from light and evaporation and incubated at room temperature for one hour, then read on a TECAN Ultra Fluorescence Polarization plate reader (Research Triangle Park, NC). The IC_{50} for the PDK1 competitor was 9 nM (n = 4).

References

- Checovich, W.C., Bolger, R.E. and Burke, T. (1995) *Nature* **375:**254-6.
- Jameson, D.M. and Sawyer, W.H. (1995) *Meth. Enzymol.* **246:**283-300.
- Vanhaesebroeck, B. and Alessi, D.R. (2000) *Biochem. J.* **346:**561-76.

Proteins for Fluorescence Polarization-based Assay Kits

RECEPTORS

Product	Expression System	Part No.	Quantity	Related Products
Androgen Receptor# Ligand-binding domain, Recombinant Rat	*E. coli*	P2719	750 pmol*	Assay Coming Soon.
Estrogen Receptor-α§ Recombinant Human	Baculovirus	P2187	750 pmol*	Estrogen Receptor-α Competitor Assay Kits (P2614, P2698)
Estrogen Receptor-β§ Recombinant Human	Baculovirus	P2466	750 pmol*	Estrogen Receptor-β Competitor Assay Kits (P2615, P2700)
Estrogen Receptor-β1 Long Form, Recombinant Human	Baculovirus	P2718	750 pmol*	Estrogen Receptor-β Competitor Assay Kits (P2615, P2700)
Glucocorticoid Receptor§ Recombinant Human	Baculovirus	P2812	100 pmol*	Glucocorticoid Receptor Competitor Assay Kit, Green (P2816) Glucocorticoid Receptor Competitor Assay Kit, Red (P2893)
Progesterone Receptor B†§ Recombinant Human	Baculovirus	P2835	100 pmol*	Progesterone Receptor Competitor Assay Kit (P2895, P2896)
Progesterone Receptor‡§ Ligand-binding domain, Recombinant Human	*E. coli*	P2899	400 pmol*	Progesterone Receptor Competitor Assay Kit (P2895, P2896)

#Thioredoxin-fusion
*Active Receptor
§These receptors are included with the fluorescence polarization-based Assay Kits listed under "Related Products."
†Histidine-tagged
‡GST-fusion

Please inquire for bulk purchases of these proteins.

KINASES

Product	Expression System	Part No.	Quantity	Related Products
Cdk5/p35 Recombinant Human	Coming Soon			
Csk† Recombinant Human	*E. coli*	P2927	10 µg	Protein Tyrosine Kinase Assay Kits, Green (P2836, P2837) Protein Tyrosine Kinase Assay Kits, Red (P2882, P2883)
Cyclic AMP-dependent Protein Kinase† Catalytic Subunit, Recombinant Human	*E. coli*	P2912	10 µg	Protein Kinase C Assay Kits, Green (P2747, P2748) Protein Kinase C Assay Kits, Red (P2940, P2941)
Epidermal Growth Factor (EGF) Receptor Purified Human	A431 Cells	P2628	10 U	Protein Tyrosine Kinase Assay Kits, Green (P2836, P2837) Protein Tyrosine Kinase Assay Kits, Red (P2882, P2883)
Hck† Recombinant Human	Baculovirus	P2908	10 µg	Protein Tyrosine Kinase Assay Kits, Green (P2836, P2837) Protein Tyrosine Kinase Assay Kits, Red (P2882, P2883)
Lyn A† Recombinant Human	Baculovirus	P2906	10 µg	Protein Tyrosine Kinase Assay Kits, Green (P2836, P2837) Protein Tyrosine Kinase Assay Kits, Red (P2882, P2883)
Lyn B† Recombinant Human	Baculovirus	P2907	10 µg	Protein Tyrosine Kinase Assay Kits, Green (P2836, P2837) Protein Tyrosine Kinase Assay Kits, Red (P2882, P2883)
PDK1 Recombinant Human	Coming Soon			
PKBα/Akt1 Recombinant Human	Coming Soon			
PKCα (alpha) Recombinant Human	Baculovirus	P2232 P2227	5 µg 20 µg	Protein Kinase C Assay Kits, Green (P2747, P2748) Protein Kinase C Assay Kits, Red (P2940, P2941)
PKCβI (beta I) Recombinant Human	Baculovirus	P2291 P2281	5 µg 20 µg	Protein Kinase C Assay Kits, Green (P2747, P2748) Protein Kinase C Assay Kits, Red (P2940, P2941)
PKCβII (beta II) Recombinant Human	Baculovirus	P2254 P2251	5 µg 20 µg	Protein Kinase C Assay Kits, Green (P2747, P2748) Protein Kinase C Assay Kits, Red (P2940, P2941)
PKCγ (gamma) Recombinant Human	Baculovirus	P2233 P2228	5 µg 20 µg	Protein Kinase C Assay Kits, Green (P2747, P2748) Protein Kinase C Assay Kits, Red (P2940, P2941)
PKCδ (delta) Recombinant Human	Baculovirus	P2293 P2287	5 µg 20 µg	Protein Kinase C Assay Kits, Green (P2747, P2748) Protein Kinase C Assay Kits, Red (P2940, P2941)
PKCε (epsilon) Recombinant Human	Baculovirus	P2292 P2282	5 µg 20 µg	Protein Kinase C Assay Kits, Green (P2747, P2748) Protein Kinase C Assay Kits, Red (P2940, P2941)
PKCζ (zeta) Recombinant Human	Baculovirus	P2273 P2268	5 µg 20 µg	Protein Kinase C Assay Kits, Green (P2747, P2748) Protein Kinase C Assay Kits, Red (P2940, P2941)
PKCη (eta)† Recombinant Human	Baculovirus	P2633 P2634	5 µg 20 µg	Protein Kinase C Assay Kits, Green (P2747, P2748) Protein Kinase C Assay Kits, Red (P2940, P2941)
PKCθ (theta)† Recombinant Human	Baculovirus	P2996	5 µg	Protein Kinase C Assay Kits, Green (P2747, P2748) Protein Kinase C Assay Kits, Red (P2940, P2941)
PKC Panel Recombinant Human	Baculovirus	P2352	1 µg each	Protein Kinase C Assay Kits, Green (P2747, P2748) Protein Kinase C Assay Kits, Red (P2940, P2941)
Src, partially purified Recombinant Human	Baculovirus	P2903	10 µg	Protein Tyrosine Kinase Assay Kits, Green (P2836, P2837) Protein Tyrosine Kinase Assay Kits, Red (P2882, P2883)
Src N1 Recombinant Human	Baculovirus	P2904	10 µg	Protein Tyrosine Kinase Assay Kits, Green (P2836, P2837) Protein Tyrosine Kinase Assay Kits, Red (P2882, P2883)
Src N2 Recombinant Human	Baculovirus	P2909	10 µg	Protein Tyrosine Kinase Assay Kits, Green (P2836, P2837) Protein Tyrosine Kinase Assay Kits, Red (P2882, P2883)
ZAP-70† Recombinant Human	Baculovirus	P2782	20 µg	Protein Tyrosine Kinase Assay Kits, Green (P2836, P2837) Protein Tyrosine Kinase Assay Kits, Red (P2882, P2883)

†Histidine-tagged

Please inquire for bulk purchases of these proteins.

Beacon® 2000 Fluorescence Polarization Systems

Product No.	Description
P2300	Beacon® 2000 Fluorescence Polarization System, 110 V Standard Beacon® 2000 FP Instrument (360-700 nm range) with fluorescein filters installed (488 nm excitation, 535 nm emission), plus complete set of starter reagents and choice of Option A or Option B.
P2302	Beacon® 2000 Fluorescence Polarization System, 220 V/240 V† (See description for P2300)
P2370	Beacon® 2000 Full-Range Fluorescence Polarization System, 110 V Full-Range Beacon® 2000 FP Instrument (254-700 nm range) with fluorescein filters installed plus complete set of starter reagents (2 Kits) and choice of Option A or Option B.
P2372	Beacon® 2000 Full-Range Fluorescence Polarization System, 220 V/240 V† (See description for P2370)
P2810	Beacon® 2000 Red Range Fluorescence Polarization System, 110V
P2811*	Beacon® 2000 Red Range Fluorescence Polarization System, 220V

Option A: Low Volume Chamber (100 µL minimum volume) and Low Volume Accessory Kit* with 6 x 50 mm tubes
Option B: Standard Volume Chamber (500 µL minimum volume) and Standard Volume Accessory Kit* with 10 x 75 mm tubes

† Contact PanVera® for details on CE Mark.
* Accessory Kits include the following: 250 Beacon® Disposable Test Tubes (either 6 x 50 mm or 10 x 75 mm); Beacon® 2000 Data Manager Software, ver. 2.2; Thermal Printer (parallel); 2 rolls of Printer Paper; Printer Cable (3' parallel); Computer Cable (6'); Replacement Lamp; Literature Packet (including Instruction Manual).

Description

The Beacon® 2000 System is an ideal tool for basic research and assay development. Two versions of the System are available. Both provide variable temperature control of the sample from 6˚C to 65˚C, and a choice of sample chambers requiring a minimum of 100 µL or 500 µL volumes. They are both equipped with external filter holders, thus accommodating a wide range of different wavelength interference filters. The filter pair for use with fluorescein is provided (488 and 535 nm); please contact PanVera® for customization of filters for other fluorophores.

The Standard Beacon® 2000

The Standard Beacon® 2000 System uses a quartz-halogen lamp that yields a usable wavelength range of 360-700 nm. All of the assays described in this Chapter may be performed with the Standard Beacon® 2000. For additional capabilities in the ultraviolet range (to 254 nm), and the extended red range (to 900 nm), the Full-Range Beacon® 2000 is available.

The Full-Range Beacon® 2000

The Full-Range Beacon® 2000 System provides ultimate flexibility, with detection capabilities in the ultraviolet and visible ranges (254-700 nm). The instrument may also be ordered with an optional photomultiplier tube (PMT) that extends the emission detection range to 900 nm. The Full-Range System is equipped with both a quartz-halogen and a mercury lamp, and with Glan-Taylor prism polarizers. This extended-range system was designed for applications and fluorophores in the ultraviolet. The Full-Range System fully accommodates all applications of the Standard Beacon® 2000 System. This expanded instrument is required for fluorescence polarization studies in the ultraviolet range, in which fluorophores such as tryptophan, pyrene, dansyl chloride, and IAEDNS are used.

The Beacon® 2000 Fluorescence Polarization Instrument

Beacon® 2000 System Components

The Beacon® 2000 Fluorescence Polarization Instrument
The core component to the Beacon® System is this stand-alone, benchtop instrument which offers sensitive polarization measurement (requiring as little as 10 fmol/mL of fluorescently-labeled sample), in a single-tube format. The instrument uses standard disposable glass test tubes and its small footprint maximizes precious bench space. Fluorescein filters (488 nm excitation, 535 nm emission) are installed as standard features. The Beacon® 2000 instrument's onboard operating software lets you store up to 99 user-defined protocols and send data directly to a thermal paper printer (included) and/or exported to your personal computer (not included) for analysis. In addition to high sensitivity and precision, the Beacon® 2000 FP System provides:

• *Variable temperature control of the sample* from 6°C to 64°C (in 1°C increments), for precise measurement of temperature-dependent reactions.

• *Low sample volume* requirement, from only 100 µL to 500 µL, depending on which instrument version is ordered.

• *External filter holders* allow for easy use of other fluorophores that excite and emit within the 360-700 nm range for the standard configuration and 254-700 nm for the full-range instrument.

Beacon® 2000 Data Manager Software, PC version 2.2b (for Windows 3.1, 95, 98 or NT®) Although the Beacon® 2000 can be used as a fully functional, stand-alone instrument, the Beacon® 2000 Data Manager Software provides the means to record and tabulate data imported directly from the instrument. These data can then be easily copied and pasted into commercially available spreadsheets, such as Microsoft® Excel™ or Lotus 123®, or into a plotting/curve-fitting program. The Data Manager software also automatically displays and graphs data points in "real time" as they are acquired from the Beacon® 2000 Instrument. This feature allows you to easily monitor the progress of your experiment as it unfolds. Software requires a PC-compatible computer running Windows 3.1, 95, 98 or NT®.

External Thermal Printer (parallel port, 40-column format)

Complete Set of Starter Reagents, Including:
• Beacon® 2000 One-Step FP Standardization Kit (Product No. P2581)
• Beacon® *trp* Repressor Kit (Product No. P2202)

Beacon® 2000 Accessories and Operator's Manual

Beacon® 2000 Instrument Specifications

Highlights

High Precision: Less than 2 mP standard deviation, at 1 nM fluorescein, for 0.5 mL sample.

High Sensitivity: Accurate to 10^{-11} M fluorescein in polarization mode and 10^{-13} M fluorescein in intensity mode.

Temperature Control: Variable control of sample temperature from 6°C to 65°C in 1°C increments, ±1°C. Calibration program provided.

Sample Volume & Tube Size: Standard Volume Chamber: Minimum sample volume of 500 µL in disposable 10 × 75 mm borosilicate glass test tubes. Low Volume Chamber: Minimum sample volume 100 µL in disposable 6 × 50 mm borosilicate glass test tubes.

Operating Software: Step-by-step prompting for measurement of blanks and samples. A "Main Menu" and "Super-User Menu" are provided. Main Menu: Selections are: Run Protocol, Create Protocol, Edit Protocol, Delete Protocol, Print Protocol, Print All Protocols, Print Protocol Status, Display Control, and Open Super-User Menu. Super-User Menu: Selections are: Show Protocols Password, Delete User Protocol, Delete All Protocols, Number of Defined Protocols, Set Default Temperature, Calibrate Temperature, Set Zeta Coefficient, Set Clock Time, Exit Super-User Menu.

Read Time: Minimum 6 seconds; number of read cycles variable, for very high precision.

Programmable Protocols: Up to 99 user-defined protocols, password protected. Variables are: Select Read Mode (Static, Kinetic), Select Blank Type (No Blank, Single Blank, Before Sample, Batch), Sample Delay (0-999 sec.), Number of Average Read Cycles (1-99), Temperature Parameters (Default Temperature, Set Temperature), Select Control Type (Auto Range, Manual), Assign Data Output (To Printer, To RS232, To Printer & RS232, To Display Only), Print Protocol (Yes/No)?, Save Protocol (Yes/No)?

Output Signals and Printer: RS232 for interface with an PC-compatible computer (not included); DB25 Parallel for interface with external parallel, thermal printer (included).

Data Manager Software: Windows 3.1, 95, 98 or NT® compatible, graphical, data acquisition software provided for use on PC-compatible computer (not included).

Mechanical

Sample Format: Single-tube
Overall Dimensions: 20 1/2" x 14 3/4" x 9 1/4" (52.6 x 37.8 x 23.7 cm)
Weight: 35 lb (15.9 kg)

Electro-Optical

Excitation Illumination: Standard Instrument: High-intensity 100 Watt quartz halogen lamp for 360-900 nm excitation. Full-range Instrument: Same as standard instrument plus 100 Watt high-intensity mercury lamp for excitation from 254 nm to 420 nm.

Emission Detector: Photomultiplier tube (PMT) with selected high gain, low noise, low drift for 200 to 700 nm emission detection. An optional photomultiplier tube that increases the emission detection range to 900 nm is also available for special order.

Optics: Five quartz lens optical system focuses excitation energy onto the sample, and collects and focuses emitted fluorescent energy onto the PMT detector. Excitation and emission polarizers and filters are in collimated light path for optimum performance.

Optical Filters & Holders: Fluorescein filters (488 and 535 nm) standard, in easily accessible, external filter holders. Filter holders accommodate other wavelength filters for use with different fluorophores.

Polarizers:
Standard Instrument: Film polarizers.
Full-range Instrument: Glan-Taylor prism polarizer.

Display: Two-line LED display, variable intensity, with 24 characters/line.

Fuse Type: Type 216.316, 3.15 Amps @ 250 Volts, 5 x 20 mm, Fast-Acting Fuse.

RFI and EMI Standards: Designed to meet most standards; CE Mark pending.

Operating Environment

Power: Two configuration: 15 V, 1.6 A @ 50 Hz or 220-240 V, 2.0 A @ 50 Hz
Operating Temperature: 15°C to 25°C
Operating Humidity: 0-75%, non-condensing

Beacon® 2000 Data Manager Software

(Note: One copy of the Data Manager Software is included with each Beacon® 2000 System.)

Product No.	Quantity
P2234	2 × 3.5" Floppy Disk

Description

Although the Beacon® 2000 instrument can be used as a fully functional, stand-alone instrument, the Beacon® 2000 Data Manager Software provides the means to record and tabulate data imported directly from the instrument. These data can then be copied and pasted into commercially available spreadsheets, such as Microsoft® Excel or Lotus® 123, or into a curve-fitting program. The Data Manager Software also automatically displays and graphs data points in "real time" as they are acquired from the Beacon® 2000 Instrument. This feature allows you to easily monitor the progress of your experiment as it unfolds.

Hardware Requirements

PC-compatible computer (not provided) having Microsoft® Windows 3.1, 95, 98 or NT® installed, a free serial COM Port, at least 162 KB of RAM, 2 MB available HD space, keyboard, mouse, 3.5" floppy disk drive, and monitor. A serial cable with a 9-pin male end and a 9-pin female end is provided with the Beacon® 2000 Systems.

Beacon® 2000 Accessory Items

Sample Chambers		
Product No.	**Size**	**Minimum Volume**
P2257	Standard Volume (uses P2245, below)	500 μL
P2258	Low Volume (uses P2244, below)	100 μL
Disposable Test Tubes		
Product No.	**Size**	**Minimum Volume**
P2245	10 × 75 mm	500 μL
P2244	6 × 50 mm	100 μL

Sample Chambers

Although each Beacon® 2000 System includes one preinstalled sample chamber (size specified at time of order), optional chambers are available and easily retrofit by the user.

Disposable Test Tubes

Borosilicate Glass. Certified for use with the Beacon® 2000 Systems, 1,000 tubes/case

Beacon® 2000 Replacement Components

Product No.	Description	Quantity
P2285	Replacement Thermal Paper	10 Rolls/Pack
P2249	Replacement Lamp, Quartz-Halogen, 100 W, High Intensity	2 lamps/Pack
P2501	Replacement Lamp, Mercury, For Full-Range Beacon® 2000 System only	1 Lamp/Pack
P2248	Replacement Fuses, Type 216.316, 3.15 A @ 250 Volts	5 Fuses/Pack

Beacon® 2000 One-Step FP Standardization Kit

(Note: This kit is included with each Beacon® 2000 System purchase)

Product No.
P2581

Description

The Beacon® 2000 One-Step FP Standardization Kit is used to demonstrate characteristic high and low fluorescence polarization values of fluorescein solutions to new users of the Beacon® 2000 System. It also allows users to compare the polarization values generated by their instrument to the expected values of known solutions. No dilution is needed.

Kit Components

Beacon® 2000 High Polarization Standard, 1X	4 mL
Beacon® 2000 Low Polarization Standard, 1X	4 mL
Beacon® 2000 BGG/Phosphate Buffer	15 mL

RED (FP) Standardization Kit

Product No.
P2888

Kit Components

Red Polarization Standard (RPS)	4 mL
RPS Buffer	8 mL

Description

The Red Fluorescence Polarization (FP) Standardization Kit is used calibrate and standardize single-tube and microplate high throughput screening (HTS) instruments that measure fluorescence polarization.

Beacon® 2000 UV Standardization Kit

Product No.
P2500

Kit Components

Beacon® 2000 High Polarization Standard, 1X	4 mL
Beacon® 2000 Low Polarization Standard, 1X	4 mL
Beacon® 2000 BGG/Phosphate Buffer	15 mL

Description

The Beacon® 2000 UV Standardization Kit uses tryptophan and bovine gamma globulin (BGG) standard solutions to demonstrate that the Beacon® 2000 Full-Range instrument is operating within normal parameters in the ultraviolet range. The assay requires a 300 nm excitation filter and a 400 nm emission filter.

Beacon® 2000 *trp* Repressor Kit

(Note: This kit is included with each Beacon® 2000 System purchase)

Product No.	No. of Assays
P2202	10

Kit Components

trp Repressor Protein (TrpR)	150 µL
Fluorescein-labeled *trp Operator* (*trp*O-F)	1 mL
trp Binding Buffer	2×120 mL

Description

Using fluorescence polarization, this kit provides an easy and sensitive method for measuring the binding of *trp* repressor to a fluorescein-labeled *trp* operator oligonucleotide. The graph to the right displays multiple equilibria between *trp* repressor dimer-DNA binding and dimer-dimer binding, as measured on the Beacon® 2000 instrument.

Beacon® 2000 (FP) DNase Activity Detection Kit

Fluorescence polarization-based kit*

| Product No.	No. of Assays
P2012	100

Application

• Rapid, sensitive quantitation of DNase activity

Description

The Beacon® 2000 (FP) DNase Activity Detection Kit is a nonradioactive, homogeneous assay system used to determine the presence of DNase in biological samples, buffers, and reagents. The method requires no separation or precipitation of the reaction mixture. Measurements are taken in real time with the Beacon® 2000 Fluorescence Polarization Systems. The Beacon® 2000 Systems measure the decrease in polarization of a fluorescein-labeled DNA substrate due to the degradation by DNases.

Sensitivity

The Beacon® 2000 (FP) DNase Activity Detection Kit, used as recommended, provides sensitivity similar to a conventional radioactive DNase assay using tritium-labeled DNA.
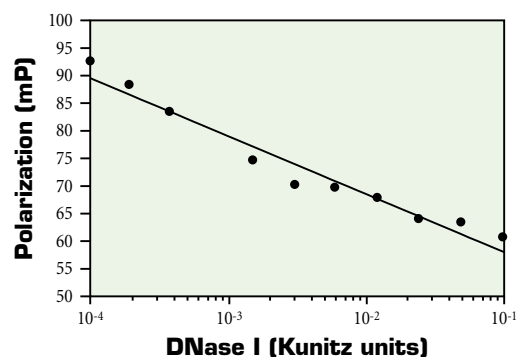
Standard curve of DNase activity.

Kit Components

Beacon® Fluorescein DNA	1 mL
Beacon® 5X DNase Assay Buffer	2×1 mL
Beacon® DNase Positive Control	100 µL
Nuclease-Free Water	10 mL
Beacon® DNase Quench Buffer	120 mL

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* The method for detecting nucleic acid cleavage utilizing a fluorescent tag in the Beacon® 2000 RNase Activity Detection Kit and the Beacon® 2000 DNase Activity Detection Kit is covered by U.S. Patent No. 5,786,139 issued to PanVera® Corporation.

Beacon® 2000 (FP) RNase Activity Detection Kit

Fluorescence polarization-based kit*

| Product No.	No. of Assays
P2115	100

Application
• Rapid, sensitive quantitation of ribonuclease activity

Description

The Beacon® (FP) RNase Activity Detection Kit is a non-radioactive assay system used to determine the presence of RNase in biological samples, buffers, and reagents. The method requires no separation or precipitation of the reaction mixture. Measurements are taken in real time with the Beacon® 2000 Fluorescence Polarization System. The Beacon® 2000 Systems measure the decrease in polarization of a fluorescein-labeled RNA substrate, due to the degradation by RNases. The nature of the FP technology eliminates the need for electrophoresis.

Sensitivity

The Beacon® 2000 (FP) RNase Activity Detection Kit is more sensitive than the conventional tritium-labeled RNA assays, when comparing 1-2 hour incubation times. With incubation times of 16 hours, the Beacon® 2000 Kit can detect as little as 1 pg of RNase.
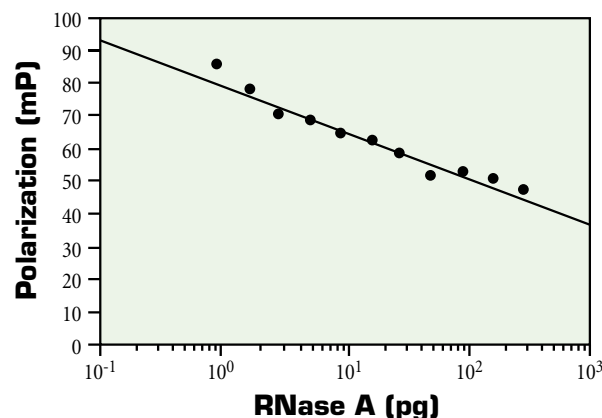
Standard curve of RNase activity.

Kit Components

Beacon® Fluorescein RNA	2×250 μL
Beacon® 5X RNase Assay Buffer	2×1.0 mL
Beacon® RNase Positive Control	100 μL
Nuclease-Free Water	10 mL
Beacon® RNase Quench Buffer	120 mL

* The method for detecting nucleic acid cleavage utilizing a fluorescent tag in the Beacon® 2000 RNase Activity Detection Kit and the Beacon® 2000 DNase Activity Detection Kit is covered by U.S. Patent No. 5,786,139 issued to PanVera® Corporation.

RNase Activity Detection Kit

Fluorescence intensity-based kit

Product No.	No. of Assays
P2011	100

Application
• Rapid, sensitive quantitation of ribonuclease activity

Description

The RNase Activity Detection Kit is a nonradioactive RNase assay system used to determine the presence of RNase in biological samples, buffers, and reagents. The system is a fluorescence-based precipitation assay which requires no gel electrophoresis. The samples to be tested for RNase activity are incubated in the presence of a fluorescein-labeled RNA (F-RNA) substrate. After TCA precipitation, the degraded (TCA soluble) RNA is quantitated by measurement of fluorescence intensity at 530 nm.

Sensitivity

The RNase Activity Detection Kit, used as recommended, provides sensitivity similar to a conventional radioactive RNase assay using tritium-labeled RNA.

Kit Components

Fluorescein RNA (5 ng/mL)	1.0 mL
5X RNase Assay Buffer	1.0 mL
Calf Thymus DNA (1.0 mg/mL)	1.0 mL
RNase Positive Control	100 μL
Nuclease-Free Water	10 mL
Beacon® Assay Buffer	100 mL

Beacon® 2000 (FP) Protease Activity Detection Kit

Fluorescence polarization-based kit

Product No.	No. of Assays
P2010	200

Application
Rapid, sensitive quantitation of protease activity

Description
This detection kit provides a sensitive, straightforward procedure for quantitating protease activity using fluorescein thiocarbamoyl (FTC)-casein, without the need for any separation, manipulation, or transfers of the reaction mixture. Measurements are taken in real time with the Beacon® 2000 Fluorescence Polarization System.

Sensitivity
The Beacon® 2000 (FP) Protease Activity Detection Kit, used as recommended, is approximately 200-fold more sensitive than the casein gel assay or casein A_{280} assays.

Kit Components
Beacon® FSE-casein 2 µg/mL	5×1 mL
Beacon® Incubation Buffer	2×120 mL
Beacon® Assay Buffer	2×120 mL
Beacon® Protease Positive Control	1 mL

Protease Activity Detection Kit

Fluorescence intensity or absorption-based kit

Product No.	No. of Assays
P2006	200

Application
Rapid, sensitive quantitation of protease activity

Description
Researchers and quality assurance laboratories often need to verify specific protease activities or to detect protease contaminations in final products. Unfortunately, many commonly used assays 1) are tedious and time-consuming to develop, 2) are not sensitive enough (e.g., casein-gel method or casein A_{280} assay), or 3) require radioactive substrates. Because of the lack of an easy, rapid, sensitive general protease assay, many scientists have foregone testing common reagents. PanVera®'s Protease Activity Detection Kit overcomes these difficulties by measuring the proteolytic degradation of fluorescein-labeled casein using absorbance at 492 nm or fluorescence, with high sensitivity and without the use of radioactive labeling.

Sensitivity
The Protease Activity Detection Kit, used as recommended, is approximately 100-fold more sensitive than either the casein-gel assay or casein A_{280} assay. The sensitivity of the Protease Activity Detection Kit can be increased as much as 35-fold by increasing the incubation time from 1 hour to 24 hours. The actual increase in sensitivity will depend on the stability of the protease to autocleavage and thermal breakdown.

Kit Components
FTC-casein in Incubation Buffer	10 mL
Incubation Buffer	10 mL
Assay Buffer	120 mL
Protease Positive Control	1 mL

Fluorescein Amine Labeling Kit

Product No.	No. of Reactions
P2058	≥36

Description
The Fluorescein Amine Labeling Kit enables the efficient conjugation of fluorescein to peptides or proteins at amino residues using fluorescein succinimidyl ester. The resulting amide bonds of fluorescein-labeled peptides (F-peptides) from succinimidyl esters are much more stable than those formed using FITC or thiol-reactive fluorophores. The procedure is optimized for maximal selectivity of N-terminal labeling. The kit contains enough reagents for a minimum of 36 different 100 µg labelings of a 2,000 Da peptide. Each labeling is done at two different label-to-peptide ratios and includes a blank. Larger peptides require less reagent, increasing the number of reactions per kit.

Kit Components
Fluorescein succinimidyl ester (FS)	6×2 mg
Dimethylsulfoxide	2×1 mL
Coupling Buffer, 10X	1 mL
Quench Buffer, 10X	1 mL

Fluorescein-C6 Amine Labeling Kit

Product No.	No. of Reactions
P2099	≥15

Description

A similar labeling kit to P2058 except that it uses a fluorescein derivative with a six-carbon spacer adjacent to the succinimidyl ester, rather than being directly conjugated to the ester. Use of the C6 spacer may minimize steric interference by the bulky fluorescein moiety in some cases.

Kit Components

Fluorescein-C6 succinimidyl ester (FXS)	3×2 mg
Dimethylsulfoxide (DMSO)	2×1 mL
Coupling Buffer, 10X	1 mL
Quench Buffer, 10X	1 mL

Bovine Serum Albumin

10%, Ultrapure Molecular Biology Grade (nonacetylated)

Product No.	Quantity
P2489	25 mL, 10% solution
P2046	100 mL, 10% solution

Applications

- Probe-based diagnostics
- DNA footprinting and gel shift assays
- PCR and restriction enzyme reactions

Description

PanVera®'s Ultrapure Bovine Serum Albumin (BSA) is rigorously tested for RNase and DNase contamination, and is the reagent of choice when RNA and DNA integrity is essential. This level of purity is achieved by chromatographic purification of native BSA and not by chemical modification with acetic anhydride. In addition, Ultrapure BSA is tested for interfering activities such as protease activity, alkaline phosphatase, peroxidase, and provides ultralow fluorescence background.

Ultrapure BSA is well-suited for applications in which acetylated BSA is not desirable, such as probe-based diagnostics, immunoblotting, receptor binding studies, antibody dilution, radioactive quenching, fluorescence polarization, enzyme stabilization, ELISA, RIA, immunofluorescence, and enzyme assays. Ultrapure BSA is aseptically processed and filtered through a 0.2 μm membrane.

Form

10% stock solution (w/v, in water), filtered through a 0.2 μm membrane.

Source

Bovine serum is of USA origin, collected at USDA-licensed and inspected abbattoirs.

Bovine Gamma Globulin

0.5% Ultrapure Molecular Biology Grade (acetylated)

Product No.	Quantity
P2045	10 mL
P2255	100 mL

Applications

- Carrier protein in fluorescent assays
- Enzyme stabilization
- Background reduction in probe-based assays

Description

PanVera®'s Ultrapure Bovine Gamma Globulin (BGG) is suitable for use as a carrier in protein precipitations and as a stabilizer in applications where small amounts of contaminating degradative enzymes can adversely affect performance. Additional purification steps have been taken to minimize absorbance and fluorescence background. This protein is tested to higher specifications for contamination than those currently supplied by other companies, including tests for protease, RNase, DNase, endonuclease, peroxidase, and ultralow fluorescence.

Applications include immunoblots, receptor binding studies, antibody dilution, radioiodination quenching, enzyme stabilization, ELISA, RIA, immunofluorescence, and enzyme assays such as reverse transcriptase, protein kinase assays, or coupled diagnostic assays.

Form

5 mg/mL solution in water, filtered through a 0.2 μm membrane

ORDERING INFORMATION

HOW TO ORDER

Orders may be placed by telephone, fax, or e-mail. When ordering please have the following information ready:

1. Billing address or Customer Number (if known)
2. Shipping address
3. Telephone number and the name of a contact
4. Purchase order number or credit card information
5. Product number and description of product

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For the latest prices, please contact PanVera® or visit our website at: www.panvera.com.

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PATENTS

The method for detecting nucleic acid cleavage utilizing a fluorescent tag in the Beacon® 2000 RNase Activity Detection Kit and the Beacon® 2000 DNase Activity Detection Kit is covered by U.S. Patent No. 5,786,139 issued to PanVera® Corporation. Other U.S. and international patents pending.

The method for detecting reverse transcriptase activity using fluorescence polarization is covered by U.S. Patent No. 6,100,039 issued to PanVera® Corporation. Other U.S. and international patents pending.

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