Fluorescence Polarization
<table>
<thead>
<tr>
<th>Contents</th>
<th>Fluorescence Polarization Technical Resource Guide • iii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trademarks and Patents</td>
<td>vi</td>
</tr>
<tr>
<td>Foreword to the Fourth Edition</td>
<td>vii</td>
</tr>
<tr>
<td>Chapter 1 - Introduction</td>
<td>1-1</td>
</tr>
<tr>
<td>Introduction</td>
<td>1-2</td>
</tr>
<tr>
<td>Theory</td>
<td>1-2</td>
</tr>
<tr>
<td>Anisotropy or Polarization?</td>
<td>1-4</td>
</tr>
<tr>
<td>The Beacon® Fluorescence Polarization System</td>
<td>1-5</td>
</tr>
<tr>
<td>High Throughput Screening (HTS) Using FP</td>
<td>1-5</td>
</tr>
<tr>
<td>Limitations of FP</td>
<td>1-7</td>
</tr>
<tr>
<td>Chapter 2 - Receptor-Ligand Binding</td>
<td>2-1</td>
</tr>
<tr>
<td>Introduction</td>
<td>2-2</td>
</tr>
<tr>
<td>Competitive Binding Assays for Nuclear Receptors</td>
<td>2-2</td>
</tr>
<tr>
<td>GST-Src Homology Domain Binding to a Fluorescein-Phosphopeptide</td>
<td>2-7</td>
</tr>
<tr>
<td>Chapter 3 - Immunoassays</td>
<td>3-1</td>
</tr>
<tr>
<td>Introduction</td>
<td>3-2</td>
</tr>
<tr>
<td>Detection of Kinase Activity Using Fluorescence Polarization (Patent Pending)</td>
<td>3-3</td>
</tr>
<tr>
<td>Tyrosine Kinase Assays</td>
<td>3-3</td>
</tr>
<tr>
<td>Serine/Threonine Kinase Assays</td>
<td>3-5</td>
</tr>
<tr>
<td>Detection of Tyrosine Phosphatase Activity Using Fluorescence Polarization (Patent Pending)</td>
<td>3-7</td>
</tr>
<tr>
<td>Protein Tyrosine Phosphatase Assay</td>
<td>3-8</td>
</tr>
<tr>
<td>Inhibition of TC PTP by Sodium Vanadate</td>
<td>3-9</td>
</tr>
<tr>
<td>Quantitation of Antigen: FP Immunoassay for Epidermal Growth Factor</td>
<td>3-10</td>
</tr>
<tr>
<td>Introduction</td>
<td>3-10</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>3-10</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>3-10</td>
</tr>
<tr>
<td>Useful Literature</td>
<td>3-12</td>
</tr>
<tr>
<td>Characterization of a Single-chain Antibody</td>
<td>3-13</td>
</tr>
<tr>
<td>Introduction</td>
<td>3-13</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>3-13</td>
</tr>
<tr>
<td>Method A (from Dandliker et al., 1981)</td>
<td>3-13</td>
</tr>
<tr>
<td>Method B (from Lundblad et al., 1996)</td>
<td>3-15</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>3-15</td>
</tr>
<tr>
<td>Chapter 4 - DNA-Protein Binding</td>
<td>4-1</td>
</tr>
<tr>
<td>DNA-Protein Binding</td>
<td>4-2</td>
</tr>
</tbody>
</table>
Chapter 5 - Degradative Assays ............................................................... 5-1
Degradative assays ................................................................. 5-2
A Quantitative Amylase Assay in Malt and Molasses Samples ................. 5-12
  Introduction ................................................................. 5-12
  Materials and Methods ...................................................... 5-12
  Results and Discussion ...................................................... 5-13
  Acknowledgments ............................................................ 5-14

Chapter 6 - DNA Hybridization and Detection ........................................ 6-1
Fluorescence Polarization Detection of DNA Hybridization ................. 6-2
  Introduction ................................................................. 6-2
  Materials and Methods ...................................................... 6-2
  Results and Discussion ...................................................... 6-3
  Useful Literature .............................................................. 6-3
Detection of Amplified DNA by Fluorescence Polarization ..................... 6-4
  Introduction ................................................................. 6-4
  Materials and Methods ...................................................... 6-4
  Results and Discussion ...................................................... 6-5
A Quantitative Reverse Transcriptase Assay Using Fluorescence Polarization 6-6
  Introduction ................................................................. 6-6
  Materials and Methods ...................................................... 6-7
  Results and Discussion ...................................................... 6-7

Chapter 7 - Theory of Binding Data Analysis ........................................ 7-1
Clark's Theory ........................................................................ 7-2
Non-specific Binding .......................................................... 7-3
Determination of Binding Constants ............................................... 7-4
  Saturation Function ......................................................... 7-6
  Klotz Plot ................................................................. 7-6
  Scatchard Analysis .......................................................... 7-7
  Non-specific Binding ......................................................... 7-7
  Negative Cooperativity ...................................................... 7-7
  Positive Cooperativity ....................................................... 7-8
  Chemical Instability at Low Concentrations ................................ 7-8
  Multiple Classes of Binding Sites ........................................... 7-8
  Hill Plot and Cooperativity .................................................. 7-9
  Non-linear, Least-Squares, Curve Fitting .................................. 7-10
  Multiple Classes of Binding Sites ........................................... 7-11
Chapter 8 - Analysis of FP Binding Data

Equilibrium and Non-equilibrium Conditions
Equilibrium Conditions
Non-equilibrium Conditions
Determination of the Association Rate Constant, kₐ
Determination of the Dissociation Rate Constant, kᵣ
Approximate Solutions for kₐ and kᵣ
Ways to Improve Experimental Results
Interchangeability of Terms
Important Points
Common Binding Experiment User Errors
Acknowledgment

Chapter 8 - Analysis of FP Binding Data

Determination of Binding Constants
Definitions
Polarization vs. Anisotropy
Relationship of Anisotropy to Bound/Free Ratio
Changes in Fluorescence Intensity and Fluorescence Lifetime
Seeing a Signal
Equilibrium Binding: Experimental Design
Incubation Time to Reach Equilibrium
Constructing a Binding Isotherm
Analysis of Binding Constants

Competition Experiments
General Considerations
Receptor-Ligand Competition Experiments
Kinetic Experiments
Determination of the Dissociation Rate Constant, kᵣ
Determination of the Association Rate Constant, kₐ
Trademarks and Patents

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Patents

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.
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 as 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

Theory

Anisotropy or Polarization?

The Beacon® 2000 Fluorescence Polarization System

High Throughput Screening (HTS) Using FP

Limitations of FP

1-2

1-2

1-4

1-5

1-5

1-7
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).

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

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.

**Equation 1.2:**

\[ P = \frac{\text{Intensity}_{\text{vertical}}^2 \times \text{Intensity}_{\text{horizontal}}}{\text{Intensity}_{\text{vertical}} \times \text{Intensity}_{\text{horizontal}}} \]

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 1-1.** 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.

**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:

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

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

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

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.

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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.

References

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.

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.

Table 1-1. Publications detailing the development of HTS assays using FP.

<table>
<thead>
<tr>
<th>Publication</th>
<th>Assay Type/Target Class</th>
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<tbody>
<tr>
<td>Deshpande et al. (1999)</td>
<td>Kinase</td>
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<tr>
<td>Parker et al. (2000)</td>
<td>Kinase</td>
</tr>
<tr>
<td>Wu et al. (2000)</td>
<td>Kinase</td>
</tr>
<tr>
<td>Parker et al. (2000)</td>
<td>Phosphatase</td>
</tr>
<tr>
<td>Parker et al. (2000)</td>
<td>Nuclear Receptors</td>
</tr>
<tr>
<td>Keating et al. (2000)</td>
<td>Protein-Protein</td>
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<tr>
<td>Lynch et al. (1997)</td>
<td>Protein-Protein</td>
</tr>
<tr>
<td>Lynch et al. (1999)</td>
<td>Protein-Protein</td>
</tr>
<tr>
<td>Wu et al. (1997)</td>
<td>Protein-Protein</td>
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<tr>
<td>Jolley (1996)</td>
<td>Protease</td>
</tr>
<tr>
<td>Levine et al. (1997)</td>
<td>Protease</td>
</tr>
<tr>
<td>Pope et al. (1999)</td>
<td>Protease</td>
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<tr>
<td>Chen et al. (1999)</td>
<td>Genomics</td>
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<tr>
<td>Pope et al. (1999)</td>
<td>Nucleic Acid</td>
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<tr>
<td>Allen et al. (2000)</td>
<td>GPCR</td>
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<tr>
<td>Banks et al. (2000)</td>
<td>GPCR</td>
</tr>
<tr>
<td>Pope et al. (1999)</td>
<td>Topoisomerase</td>
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<tr>
<td>Pope et al. (1999)</td>
<td>Cytokine Receptors</td>
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<tr>
<td>Li et al. (2000)</td>
<td>Transferase</td>
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<tr>
<td>Zhao et al. (1999)</td>
<td>Antimicrobials</td>
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<tr>
<td>Kauvar et al. (1995)</td>
<td>Protein-fluorescent probe binding</td>
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<tr>
<td>Sportsman et al. (1997)</td>
<td>Protein-fluorescent probe binding</td>
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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>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Fluorescence Intensity (FLINT)</td>
<td>• Simple</td>
<td>• No information for quality control</td>
</tr>
<tr>
<td></td>
<td>• Suitable for fluorogenic assays</td>
<td>• Sensitive to inner-filter and auto-fluorescence interference</td>
</tr>
<tr>
<td></td>
<td>• Readily miniaturized</td>
<td></td>
</tr>
<tr>
<td>Fluorescence Polarization (FP)</td>
<td>• simple, predictive</td>
<td>• local motion effects</td>
</tr>
<tr>
<td></td>
<td>• insensitive to inner-filter effects</td>
<td>• suitability limited by lifetime of dye, ligand size, and molecular weight change</td>
</tr>
<tr>
<td></td>
<td>• ratiometric technique</td>
<td>• can suffer from auto-fluorescence</td>
</tr>
<tr>
<td></td>
<td>• suitable for small (&lt;10 kDa) ligands</td>
<td></td>
</tr>
<tr>
<td>Fluorescence Resonance Energy Transfer (FRET)</td>
<td>• suitable for short inter/intramolecular distances (&lt;5 nm)</td>
<td>• requires multiple labels</td>
</tr>
<tr>
<td></td>
<td>• range of available donors and acceptors</td>
<td>• sensitivity to inner-filter and auto-fluorescence interference</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• limited to short distances to obtain high signal changes</td>
</tr>
<tr>
<td>Time-Resolved Energy Transfer (TR-FRET)</td>
<td>• robust</td>
<td>• requires multiple complex labels</td>
</tr>
<tr>
<td></td>
<td>• suitable for long distances (5–10 nm)</td>
<td>• limited choice of donors/acceptors</td>
</tr>
<tr>
<td></td>
<td>• reduced autofluorescence interference</td>
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</tbody>
</table>

CHAPTER 2

Receptor-Ligand Binding

Introduction .......................................................................................................................... 2-2
Competitive Binding Assays for Nuclear Receptors ....................................................... 2-2
GST-Src Homology Domain Binding to a Fluorescein-Phosphopeptide ........................ 2-7
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 fluorescein-labeled peptides).

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 (IC₅₀) of two or more unlabeled ligands or, when the Kᵢ of the labeled ligand is known, calculate the Kᵢ of the unlabeled ligands. The great benefit of competition experiments is the ability to determine binding affinities without requiring labeling the ligand under study.

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

Analysis of the competition binding curve yields IC₅₀ values, which is the concentration of unlabeled ligand necessary to displace 50% of the tracer from the receptor. IC₅₀ 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 IC₅₀ 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]/Kᵯ ratio is at least 1, so that the starting polarization value will represent at least 50% of the maximal shift. Note that IC₅₀ values determined will be greater than the actual Kᵯ values. See Chapter 8 for a detailed discussion of the relationship between IC₅₀ and Kᵯ.

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 IC₅₀ can be determined.

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.
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 unphosphorylated 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.

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 IC50 values are not precisely comparable to those in Figure 2-2, due to slight differences in receptor concentrations.

CHAPTER 3

Immunoassays

Introduction ........................................................................................................ 3-2
Detection of Kinase Activity Using Fluorescence Polarization ................................................................. 3-3
Tyrosine Kinase Assays .................................................................................................................. 3-3
Serine/Threonine Kinase Assays ........................................................................................................ 3-5
Detection of Tyrosine Phosphatase Activity Using Fluorescence Polarization ........................................... 3-7
Protein Tyrosine Phosphatase Assay ..................................................................................................... 3-8
Inhibition of TC PTP by Sodium Vanadate ............................................................................................ 3-9
Quantitation of Antigen: FPIA for Epidermal Growth Factor ................................................................... 3-10
Introduction .................................................................................................................................... 3-10
Materials and Methods ....................................................................................................................... 3-10
Results and Discussion ........................................................................................................................ 3-10
Useful Literature ............................................................................................................................... 3-12
Characterization of a Single-chain Antibody ....................................................................................... 3-13
Introduction .................................................................................................................................... 3-13
Materials and Methods ....................................................................................................................... 3-13
Method A (from Dandliker et al., 1981) .............................................................................................. 3-13
Method B (from Lundblad et al., 1996) .............................................................................................. 3-15
Results and Discussion ........................................................................................................................ 3-15
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 32P 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 throughput 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.

References:
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:

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 MgCl₂, 5 mM MnCl₂, 50 µM Na₃VO₄, 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-phospho-tyrosine 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 Kᵅ 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.
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-phosphoserine 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-

---

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 MgCl₂, 5 mM MnCl₂, 50 µM Na₃VO₄, 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.

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Figure 3-3

<table>
<thead>
<tr>
<th>[Enzyme] (ng)</th>
<th>% Phosphorylation</th>
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<tr>
<td>0</td>
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<td>25</td>
<td>75</td>
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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) can be used to determine IC\textsubscript{50} values for inhibitors of specific serine/threonine kinases such as staurosporine, a potent inhibitor of PKC isoforms (Tamaoki \textit{et al.}, 1986). Briefly, in a round-bottom, black 96-well plate, PKC isoforms (15 ng PKC\textalpha, 300 ng PKC\textbeta\textI, 300 ng PKC\textbeta\textII, 17 ng PKC\textgamma, 117-ng PKC\textdelta, or 65 ng PKC\textepsilon) 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\textsubscript{2}, 100 µM CaCl\textsubscript{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\textsubscript{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 amount of free tracer 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-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.

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 Na₃VO₄ (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 Na₃VO₄.

**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 phosphopeptides 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 Na₃VO₄. In both cases, no dephosphorylation occurred and the polarization remained high throughout the experiment.
Inhibition of TC PTP by Sodium Vanadate

To demonstrate the usefulness of an FP-based phosphatase assay in screening for phosphatase inhibitors, the IC<sub>50</sub> value for Na<sub>3</sub>VO<sub>4</sub> and TC PTP was determined in Figure 3-7. In a black, 96-well plate (DYNEX; Chantilly, VA), Na<sub>3</sub>VO<sub>4</sub> (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<sub>3</sub>VO<sub>4</sub> 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<sub>50</sub> of the ubiquitous phosphatase inhibitor Na<sub>3</sub>VO<sub>4</sub> was determined to be 4 nM for TC PTP.

Figure 3-7. The IC<sub>50</sub> value for Na<sub>3</sub>VO<sub>4</sub> and TC PTP was determined in the following manner: In a 96-well plate, Na<sub>3</sub>VO<sub>4</sub> 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<sub>3</sub>VO<sub>4</sub> concentration 2-fold. Each reaction was incubated for 15 minutes. At the end of the incubation, 10 µL of 550 µM Na<sub>3</sub>VO<sub>4</sub> 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<sub>50</sub> of approximately 4 nM.
Quantitation of Antigen: Fluorescence Polarization Immunooassay 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.](image)

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


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 (FscAb)
- 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 (FscAb) 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., Qf ≠ Qb). Both methods are described.

Method A (from Dandliker et al., 1981)

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

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

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

Solving for M:

\[ M = \frac{\left(\frac{(A - A_f)}{(A_b - A)}\right)\left(\frac{Q_f}{Q_b}\right)(A_b) + A_f}{1 + \left(\frac{(A - A_f)}{(A_b - A)}\right)\left(\frac{Q_f}{Q_b}\right)} \]

\( A, A_f \) and \( Q \) 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 than 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, \( Q \) is determined by plotting the values of \( Q \) (the observed intensity) vs. \( (Q - Q_b) / \text{receptor concentration} \). The \( y \)-intercept is \( Q_b \) (see Figure 3-10).

Equation 3.3:

\[ Q = Q_b + \frac{Q_f - Q}{\text{Receptor Concentration}} \]

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 \( [(Q_f)(A - A_f)] / [(Q)(\text{receptor concentration})] \). The \( y \)-intercept of this plot is \( A_b \) (see Figure 3-11).

Equation 3.4:

\[ A = A_b - \left[ \frac{(Q_f)(A - A_f)}{(Q_b)(\text{Receptor Concentration})} \right] \]
Method B (from Lundblad et al., 1996)

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

\[
\text{Corrected } F_b = \frac{A - A_f}{(A_b - A)(Q_b/Q_f) + A - A_f}
\]

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, $Q_b$, $A_b$, $Q_f$, and $A_f$ need to be determined. As described in Method A, $Q_b$ and $A_b$ are measurements of the free tracer in the absence of its binding partner, and $Q_f$ and $A_f$ 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).
Table 3-1. Intensity, antibody concentrations, observed millianisotropy values and corrected millianisotropy values.

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<th>Q</th>
<th>[mAb] (nM)</th>
<th>mA</th>
<th>Corrected mA</th>
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Figure 3-12. Equilibrium Binding Isotherms of Fluorescein to Anti-fluorescein Antibody.
CHAPTER 4
DNA-Protein Binding

DNA-Protein Binding ................................................................. 4-2
Introduction .................................................................................. 4-2
Binding of hER to a Fluorescein-labeled ERE .......................... 4-3
Binding of TrpR to a Fluorescein-labeled trpO ......................... 4-4
Binding of NF-IL6 to the Rat Angiotensinogen Promoter ....... 4-6
Acknowledgment ....................................................................... 4-7
Temperature-dependent Binding of TBP to the TATA Box .... 4-7
Acknowledgment ....................................................................... 4-8
Considerations for DNA-Protein Binding Studies ................ 4-9
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.

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:

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):

\[
\text{ERE1-F:} \quad 5' - \text{CGATCAGATTAGAGCTGATGCCTCGGTCACTGTGACCCAAACCCTTTA-3'} \\
\text{ERE2-F:} \quad 3' - \text{GCCTAGACTACGAGGCCAGACACTGGTCTGAGTTAAT-P-5'}
\]

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 4-1. 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).
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:

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-ATCGAACTAGTTAACTAGTACGAA - 3'
- $Trp25-A$: 3' - TAGCTTGAATCAATTGATCAGCCTT - 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
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 x 75 mm disposable borosilicate test tubes (Invitrogen Part No. P2245). The trpO-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 trpO-F is free and has a low anisotropy (mA = 70). As the TrpR concentration increases, a greater fraction of the trpO-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 trpO-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. trpO-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.

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, double-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, Figure 4-5) and low affinity site (K_d = 8400 nM).

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).

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* Invitrogen Corporation • 501 Charmary Drive • Madison, WI 53719 • USA • www.invitrogen.com/drugdiscovery
Acknowledgment
We would like to thank Dr. Allan Brasier and Dr. Amalendra Kumar at the Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, TX, for their gift of the purified TCD and D14 peptides and APRE M6 DNA oligonucleotides.

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.

Figure 4-6. Binding Isotherm of TBP to TATA Box Consensus Sequence. Various concentrations of recombinant ψTBP 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

We would like to thank Dr. Robert Roeder (Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, NY) for his gift of the TATA box binding protein and TATA oligonucleotide.
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.

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.

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Total 26,232
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 ................................................................. 5-2
A Quantitative Amylase Assay in Malt and Molasses Samples .................................................. 5-12
Introduction .............................................................................. 5-12
Materials and Methods ............................................................ 5-12
Discussion ................................................................................. 5-13
Acknowledgments ..................................................................... 5-14
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).

References:
A Quantitative Amylase Assay in Malt and Molasses Samples

Introduction
Proteases, amylases, and lipases each play important roles in the processing of dairy products, stachess 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 × 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.
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.

References

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.

<table>
<thead>
<tr>
<th>Malt Barley</th>
<th>Change in polarization (mP)</th>
<th>α-amylase activity (20 DU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M 81</td>
<td>66</td>
<td>37.8</td>
</tr>
<tr>
<td>ND 13300</td>
<td>63</td>
<td>39.8</td>
</tr>
<tr>
<td>Stander</td>
<td>60</td>
<td>36.7</td>
</tr>
<tr>
<td>Morex</td>
<td>58</td>
<td>34.2</td>
</tr>
<tr>
<td>M 75</td>
<td>57</td>
<td>36.1</td>
</tr>
<tr>
<td>Excel</td>
<td>55</td>
<td>34.8</td>
</tr>
<tr>
<td>Barbless</td>
<td>40</td>
<td>24.5</td>
</tr>
<tr>
<td>ND 14161</td>
<td>41</td>
<td>28.7</td>
</tr>
<tr>
<td>Y3250-6</td>
<td>39</td>
<td>28.6</td>
</tr>
<tr>
<td>SM 90514</td>
<td>27</td>
<td>21.4</td>
</tr>
</tbody>
</table>

### Table 5-3. Amylase Activity in Molasses Samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Change in Polarization (mP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (zero point)</td>
<td>268</td>
</tr>
<tr>
<td>Positive control (very high activity)</td>
<td>70</td>
</tr>
<tr>
<td>Sample I (&gt;60 Units/kg)</td>
<td>158</td>
</tr>
<tr>
<td>Sample IV (20 Units/kg)</td>
<td>230</td>
</tr>
<tr>
<td>Sample V (&lt;60 Units/kg)</td>
<td>199</td>
</tr>
</tbody>
</table>
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:

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 cooled 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. These changes in polarization are due to the changes in the temperature and viscosity of the solutions, and not due to hybridization.

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


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).

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.

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 .................................................. 7-2
Non-specific Binding ............................................. 7-3
Determination of Binding Constants ...................... 7-4
  Saturation Function .......................................... 7-6
  Klotz Plot ..................................................... 7-6
Scatchard Analysis .............................................. 7-7
  Non-specific Binding .......................................... 7-7
  Negative Cooperativity ...................................... 7-7
  Positive Cooperativity ...................................... 7-8
  Chemical Instability at Low Concentrations .......... 7-8
  Multiple Classes of Binding Sites ....................... 7-8
Hill Plot and Cooperativity ................................... 7-9
Non-linear, Least-Squares, Curve Fitting .................. 7-10
Multiple Classes of Binding Sites ......................... 7-11

Competition in Binding ....................................... 7-12
Equilibrium and Non-equilibrium Conditions ............ 7-14
  Equilibrium Conditions .................................... 7-14
  Non-equilibrium Conditions .............................. 7-14
    Determination of the Association Rate Constant .... 7-14
    Determination of the Dissociation Rate Constant .... 7-15
  Approximate Solutions for $k_a$ and $k_d$ .............. 7-16
Ways to Improve Experimental Results .................... 7-17
  Interchangeability of Terms ............................. 7-17
  Important Points ............................................ 7-17
  Common Binding Experiment User Errors ................ 7-17
Acknowledgment .................................................. 7-18
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 unimolecular.
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; unbound) form or bound to the receptor (B).

Under these assumptions, at equilibrium:

Equation 7.1: Receptor\text{free} + Ligand\text{free} \xleftrightarrow{k_1}{k_1^{-1}} \text{Receptor:Ligand} \xrightarrow{k_e} \text{effect}

where $k_1$ and $k_1^{-1}$ 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:

Equation 7.2: \frac{[\text{Ligand\text{free}}][\text{Receptor\text{free}}]}{[\text{Receptor:Ligand}]} = \frac{[\text{L}_F][\text{R}_F]}{[\text{B}]} = \frac{k_1^{-1}}{k_1} = K_d

We can rewrite Equation 7.2 as:

Equation 7.3: \frac{[\text{L}_F][\text{R}_T - \text{B}]}{[\text{B}]} = 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:

Equation 7.4: \frac{B}{R_T} = \frac{L_F}{(K_d + L_F)}

which is the equation for a rectangular hyperbola with horizontal asymptote corresponding to 100% saturation of $R_T$, such that $[\text{bound}] = [\text{receptor}]$, 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).
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:

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

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 $K_{nsb} >>> F$, Equation 7.5 reduces to Equation 7.6:

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

The binding to the second site therefore appears linear (unsaturable). When the second site $K_{nsb}$ 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 $K_{nsb}$ 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).

**Equilibrium binding experiment:** The concentration of one of the binding agents (usually the receptor) is kept constant and below the \( K_r (R/K_r < 0.1 \text{ or lower}) \). 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 \( K_r \) to 100-fold above the \( K_r \). 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 \( K_r \)). 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 \( K_r \)). 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.

---

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 \( L_I \) leads to:

\[
\text{Equation 7.7: } \frac{R_T \times (L_T - B)}{K_d + (L_T - B)} = B
\]

Solving for \( B \), one real solution for the quadratic is:

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

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.
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 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:

**Equation 7.9:** \[ \frac{B}{L_F} = \frac{-B}{K_d} + \frac{R_T}{K_d} \]

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.

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).

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.
Figure 7-6. A Scatchard curve indicating the presence of negative cooperativity.

Positive Cooperativity

A concave-down curve that intersects the origin is indicative of positive cooperativity (Figure 7-7). The maxima occurs at: \( f = \frac{n-1}{n} \), where \( n \) is the slope of the Hill plot (explained on page 7-10) and \( f \) = fractional occupancy.

Figure 7-7. The Scatchard plot indicating positive cooperativity.

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.

Figure 7-8. A Scatchard plot depicting that the ligand is breaking down during the experiment.

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).

Figure 7-9. This Scatchard analysis indicates the presence of multiple binding sites on the receptor.
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 O₂-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: \[ f_{\text{fractional occupancy}} = \frac{L_F^n}{K_d + L_F^n} \]

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 \( \log \frac{B}{R_T - B} \) 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>
<thead>
<tr>
<th>Table 7-1. Hill Slope Effects.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hill Slope</td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>If slope = 1, there is a single class of binding sites</td>
</tr>
<tr>
<td>If slope ≠ 1 and is an integer, multiple non-interacting binding sites</td>
</tr>
<tr>
<td>If slope ≠ 1 and is fractional, you have cooperativity:</td>
</tr>
<tr>
<td>If slope &gt;1, Positive cooperativity</td>
</tr>
<tr>
<td>If slope &lt;1, Negative cooperativity</td>
</tr>
</tbody>
</table>
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 $t$)/$B_0$ (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.

---

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).

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 plot, the composite curve will appear to be a normal hyperbola, but the apparent $K_d$ is actually equal to \( \left( \frac{K_d1 \times K_d2}{1} \right)^{1/2} \). 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:

$$ B = \frac{R_{T1} \times L_F}{K_{d1} + L_F} + \frac{R_{T2} \times L_F}{K_{d2} + L_F} $$

This model can be complicated more by the addition of a linear NSB term (resulting in a total of five parameters: $R_0$, $K_p$, $R_{ns}$, $K_{ns}$ 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 $K_{ns} >> L$, then the second site will collapse into a NSB component.

---

<table>
<thead>
<tr>
<th>Plots</th>
<th>Identifying Feature</th>
</tr>
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<tbody>
<tr>
<td>B vs. LF plot</td>
<td>A polynomial of $n + 1$ degrees being the sum of two hyperbolas</td>
</tr>
<tr>
<td>Scatchard plot</td>
<td>Concave-up curve (Figure 7-7)</td>
</tr>
<tr>
<td>Hill plot</td>
<td>Slope not equal to 1</td>
</tr>
<tr>
<td>Klotz plot</td>
<td>Multiple inflection points if the $K_i$ values differ enough</td>
</tr>
</tbody>
</table>

Table 7-2. Characteristics of Multiple Binding Sites.
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 note that the IC$_{50}$ is not necessarily equal to the KI.](image)

Historically, the affinity of the inhibitor for the receptor has been derived using the Cheng and Prussoff (1973) Corrections:

**Equation 7.13:**

$$ f_i = \frac{I}{I + K_i(1 + L_T/K_d)} $$

and

**Equation 7.14:**

$$ f = \frac{L_T}{K_d(1 + I/K_i) + L_T} $$

where:

- $f_i$ = the fractional inhibition
- $f$ = fractional occupancy or saturation
- $L_T$ = total concentration of the labeled ligand
- $I$ = Added concentration of the labeled ligand (competitor/inhibitor)
- $K_i$ = dissociation constant for labeled ligand
- $K_i$ = dissociation constant for the unlabeled ligand

---

When the value of \( f \) or \( f' \) is equal to 0.5, Equations 7-9 and 7-10 can be simplified to:

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

and

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

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., \([\text{Total}] \approx [\text{Free}]\)). 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.

\[
\text{Equation 7.17: } K_i = \frac{IC_{50}}{1 + L_T(y_0 + 2)} - K_d \left( \frac{y_0}{y_0 + 2} \right)
\]

where \( y_0 \) is 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., \( \text{Free} = \text{Total} - \text{Bound} \)). 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 \( IC_{50} \).

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

---

Rearranging to solve for $K_i$:

\[ \text{Equation 7.19:} \quad K_i = \frac{B \times I \times K_d}{(L_T \times R_T) + B \times (-R_T - L_T + B - K_d)} \]

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.

**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:

\[ \text{Equation 7.20:} \quad (R_T - B) + (L_T - B) \xrightarrow{k_1} B \xrightarrow{k_{-1}} B \]

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, $\delta t$, the concentration of bound ligand (i.e., receptor- ligand complex) will change by a small amount, $\delta B$, and therefore the following equation applies:
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:

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

where $m = (k_1)(B' - B_e)$, $C$ (the integration constant) = $\ln(B'/B_e)$, and $B_e$ is the amount bound at equilibrium. When $t = 0$, $B_i = 0$, 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, $k_1$ 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 $k_1$.

**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 $k_1$ and $K_d$, $k_{-1}$ can be calculated as $k_{-1} = (K_d/k_1)$.

**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:

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

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


$T_{1/2}$ 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).

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:

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

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

\[ \text{Equation 7.26: } \frac{\ln(2)}{T_{1/2}} = k_1 L_T + k_{-1} \]

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 $\log (B_t - B_0)/B_e$ vs. time.
4. Determine the $T_{1/2}$ for each curve (point at which $\log (B_t - B_0)/B_e = 0.5$).
5. Plot $\ln(2)/T_{1/2}$ vs. $L_T$. 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

We are deeply indebted to Dr. Antonio Colas (University of Wisconsin-Madison) for his help and advice during the preparation of this Chapter. Dr. Colas’ unpublished monographs on the analysis of binding data were an invaluable resource. The helpful discussions with Terry Kenakin, David Rodbard, and Stephane Swillens were greatly appreciated.
CHAPTER 8
Analysis of FP Binding Data

Determination of Binding Constants .................................................................................. 8-2
Definitions ............................................................................................................................ 8-2
Polarization vs. Anisotropy ................................................................................................. 8-2
Relationship of Anisotropy to Bound/Free Ratio ............................................................... 8-3
Changes in Fluorescence Intensity and Fluorescence Lifetime ........................................ 8-4
Seeing a Signal ................................................................................................................... 8-4
Equilibrium Binding: Experimental Design ...................................................................... 8-6
Incubation Time to Reach Equilibrium ........................................................................... 8-6
Constructing a Binding Isotherm ...................................................................................... 8-7
Analysis of Binding Constants .......................................................................................... 8-7

Competition Experiments ................................................................................................. 8-10
General Considerations ...................................................................................................... 8-10
Receptor-Ligand Competition Experiments ....................................................................... 8-10

Kinetic Experiments ......................................................................................................... 8-11
Determination of the Dissociation Rate Constant, $k_1$ .................................................... 8-11
Determination of the Association Rate Constant, $k_a$ ....................................................... 8-12
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 the 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):

\[
\left( \frac{1}{P} - \frac{1}{3} \right)^{-1} = \sum_{i=1}^{n} f_i \left( \frac{1}{P_i} - \frac{1}{3} \right)^{-1}
\]

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

\[
A = \sum_{i=1}^{n} (f_i)(A_i)
\]

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.

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:

Equation 8.3:  \[ A = \frac{2 \times P}{3 - P} \]

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:

Equation 8.4:  \[ A = F_f A_f + F_b A_b \]

where:  
\[ F_f + F_b = 1 \]

\[ A = \text{observed anisotropy value} \]
\[ F_f = \text{fraction of fluorescent ligand that is free} \]
\[ F_b = \text{fraction of fluorescent ligand that is bound} \]
\[ A_f = \text{anisotropy of the free fluorescent ligand} \]
\[ A_b = \text{anisotropy of the bound fluorescent ligand} \]
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 $A_{free} = 60 \text{ mA}$ (mA = millianisotropy units) and $A_{bound} = 160 \text{ mA}$, at a $mA = 110$, half of the fluorescent ligands will be bound and half will be free. At $A = 135 \text{ mA}$, 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 an 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:

$$B = \frac{L_T + K_d + R_T - \sqrt{[(L_T + K_d + R_T)^2 - 4L_TR_T]}}{2}$$

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

The explicit solution of $B$ in terms of $R$, and $L$, and the obvious interexchangeability of $R$, and $L$, 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.
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 $[receptor] = 10 \times K_i$ so that 90% of the ligand is bound, but only 9% of the receptor; Table 8-2).

<table>
<thead>
<tr>
<th>Ligand Concentration ([L]/$K_d$)</th>
<th>Receptor Concentration ([L]/$K_d$)</th>
<th>% Ligand Bound at Equilibrium</th>
<th>% Receptor Bound at Equilibrium</th>
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<tr>
<td>0.01</td>
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<td></td>
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<td>98</td>
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</table>
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 millianisotropy) 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: \[
\text{[Ligand Bound]} = L_T \times \frac{A - A_f}{A_b - A_f} = B
\]

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:

\[
\text{Equation 8.7: } B = \frac{R_F \times L_T}{K_d + R_F}
\]

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

\[
\text{Equation 8.8: } B = \frac{L_T(R_T - B)}{K_d + (R_T - B)}
\]
One solution for $B$ in Equation 8.8 is:

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

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:**

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

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

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

---

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, $k_d$

The calculation of the dissociation rate constant ($k_d$) 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 $-k_d$. When mA_{obs} = 0.5 mA_{max}, then $k_d = 0.693/T_{1/2}$, 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:

**Equation 8.13:**

$$B_t = B_e \times \{1 - e^{-(k_1 R_T + k_{-1})t}\}$$

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

**Equation 8.14:**

$$\frac{\ln(2)}{T_{1/2}} = k_1 R_T + k_{-1}$$

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

Estrogen Receptor–α and –β Competitor Assay Kits . . . . 9-2
Progesterone Receptor Competitor Assay Kits ............. 9-3
Glucocorticoid Receptor Competitor Assay Kit, Green . . 9-4
Glucocorticoid Receptor Competitor Assay Kit, Red .. 9-5
Tyrosine Kinase Assay Kits, Green and Red ............. 9-6
Ser/Thr Kinase Assay Kits, Crosstide .................... 9-7
Protein Kinase C Assay Kits, Green ....................... 9-8
Protein Kinase C Assay Kits, Red ......................... 9-9
Serine Kinase Assay Kits, IκB-α pSer 32 and pSer 36 . . . 9-10
CDK Assay Kits, Rbsup annoyed .......................... 9-11
Threonine Kinase Assay Kits, PDK 1 ....................... 9-12
Proteins for Fluorescence Polarization-based Assay Kits . . 9-13
Beacon® 2000 Fluorescence Polarization Systems ......... 9-14
Beacon® 2000 System Components ...................... 9-15
Beacon® 2000 Instrument Specifications .................. 9-15
Beacon® 2000 Data Manager Software ................... 9-16
Beacon® 2000 Accessory Items ......................... 9-16
Beacon® 2000 Replacement Components ................ 9-16
Beacon® 2000 One-Step FP Standardization Kit ........ 9-16
Red (FP) Standardization Kit .............................. 9-17
Beacon® 2000 UV Standardization Kit .................... 9-17
Beacon® 2000 trp Repressor Kit .......................... 9-17
Beacon® 2000 (FP) DNase Activity Detection Kit ....... 9-17
Beacon® 2000 (FP) RNase Activity Detection Kit ....... 9-18
RNase Activity Detection Kit .............................. 9-18
Beacon® 2000 (FP) Protease Activity Detection Kit .... 9-19
Protease Activity Detection Kit ............................ 9-19
Fluorescein Amine Labeling Kit .......................... 9-19
Fluorescein-C6 Amine Labeling Kit ...................... 9-20
BSA ...................................................... 9-20
BGG ...................................................... 9-20
Ordering Information ....................................... 9-21
Trademarks and Patents ................................... 9-22
Disclaimers .................................................. 9-22
### Applications
- Drug discovery, endocrine disruptor screening and steroid biochemistry research
- Nonradioactive measurement of estrogen receptor binding capacity by competition
- High-throughput screening
- Measurement of IC₅₀ 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

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<tr>
<th>Product No.</th>
<th>Quantity</th>
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</tr>
<tr>
<td>P2615</td>
<td>1.0 × 750 pmol</td>
</tr>
</tbody>
</table>

Please inquire for bulk purchases.

### Determination of IC₅₀ 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. IC₅₀ 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**

**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 IC₅₀ 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 IC₅₀ value was determined for each competitor. Progesterone is the most potent competitor with an IC₅₀ value of 25 nM, followed by the anti-progestin RU486 with an IC₅₀ of 60 nM. Testosterone and dexamethasone were much weaker competitors with IC₅₀ 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

Please inquire for bulk purchases.

**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 IC₅₀ 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**
Glucocorticoid Receptor Competitor Assay Kit, Green

**Product No.** P2816  
**Quantity** 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 IC₅₀ 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.

**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 Na₂MoO₄, 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 IC₅₀ and Relative Binding Affinity (RBA) for each steroid was determined from the average of 10 plate reads. Note that the RBAs 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 IC₅₀ values obtained with various steroids is shown below.

<table>
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<th>Competitive Ligand</th>
<th>Room Temperature</th>
<th>RBA</th>
<th>4°C (no peptide)</th>
<th>RBA</th>
</tr>
</thead>
<tbody>
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<td>9.9 100</td>
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<tr>
<td>Cortisol</td>
<td>30.5 38</td>
<td>35.7 28</td>
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<td>Testosterone</td>
<td>1230 0.93</td>
<td>2710 0.37</td>
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<td>β-Estradiol</td>
<td>1910 0.60</td>
<td>6810 0.15</td>
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<tr>
<td>Beclomethasone monopropionate</td>
<td>6.9 167</td>
<td>4.7 211</td>
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<tr>
<td>Fluocinonide</td>
<td>40.7 28</td>
<td>50.7 19</td>
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<td>Prednisone</td>
<td>670 1.71</td>
<td>1560 0.63</td>
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<td></td>
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</tbody>
</table>

**References**

**Bibliography**
Glucocorticoid Receptor Competitor Assay Kit, Red

**Applications**
- Ideal for drug discovery and steroid biochemistry research
- Nonradioactive measurement of glucocorticoid receptor binding capacity by competition
- High-throughput screening
- Measurement of IC₅₀ 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 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 x 25 pmol
- GR Screening Buffer, 10X 2 x 1 mL
- GR Stabilizing Peptide, 10X 2 x 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 Na₂MoO₄, 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 IC₅₀ for each steroid was determined from the average of 10 plate reads.

**Bibliography**
### Applications
- Signal transduction research and drug discovery
- Nonradioactive detection of tyrosine kinases
- High-throughput screening
- Quantitative measurement of inhibitor IC\textsubscript{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\textsuperscript{®}'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

<table>
<thead>
<tr>
<th>Kit Components</th>
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<tbody>
<tr>
<td><strong>P2836</strong></td>
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<tr>
<td>Anti-Phosphotyrosine Antibody, 10X</td>
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<tr>
<td>PTK Green Tracer, 10X</td>
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<td>PTK Competitor</td>
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<td>PTK Quench Buffer</td>
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<td>FP Dilution Buffer</td>
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<td><strong>P2837</strong></td>
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<tr>
<td>Anti-Phosphotyrosine Antibody, 10X</td>
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<td>PTK Red Tracer, 10X</td>
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<td>PTK Quench Buffer</td>
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<td>PTK Competitor</td>
</tr>
<tr>
<td>PTK Quench Buffer</td>
</tr>
<tr>
<td>FP Dilution Buffer</td>
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</table>

### Competition Curve Using Phosphotyrosine Peptide in PanVera\textsuperscript{®}'s Tyrosine Kinase Assay Kits, Green and Red.

- The IC\textsubscript{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:

<table>
<thead>
<tr>
<th>Peptide Substrate Sequence</th>
<th>Enzymes That Utilize These Substrates</th>
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</thead>
<tbody>
<tr>
<td>poly(Glu,Tyr)\textsubscript{16}</td>
<td>Zap70, Erk, Receptor, pp60 c-Src, Src N1, Src N2, Lek</td>
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<tr>
<td>ADELYLIPQ</td>
<td>EG Frceptor</td>
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<td>DLPLIPDO</td>
<td>EG Frceptor</td>
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<tr>
<td>KVKEQDQTVV</td>
<td>Fyn, Lck, lyn, pp60 c-Src</td>
</tr>
<tr>
<td>RRRYETD</td>
<td>Insulin Receptor</td>
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<tr>
<td>ELYLIPQ</td>
<td>EG Frceptor</td>
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<td>ELYSMOEI</td>
<td>EG Frceptor</td>
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<td>QLEESAM</td>
<td>H-ras, Jak2</td>
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<td>S/LQAAYLRAVPQ</td>
<td>EG Frceptor</td>
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<tr>
<td>SLNPIQPPQI</td>
<td>EG Frceptor</td>
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<td>SVNNPV5HQPNL</td>
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<td>HTDDEMGTGYVTR</td>
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PanVera\textsuperscript{®} Corporation • 501 Charmony Drive • Madison, WI 53719 • USA
Ser/Thr Kinase Assay Kits, Crosstide

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<tr>
<td><strong>P2884</strong></td>
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<tr>
<td>Ab, 4X, Crosstide Assay</td>
</tr>
<tr>
<td>Tracer, 10X, Crosstide Assay</td>
</tr>
<tr>
<td>Competitor, Crosstide Assay, 5 µM</td>
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<tr>
<td>FP Dilution Buffer</td>
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<tr>
<td>Kinase Quench Buffer</td>
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<tr>
<td><strong>P2885</strong></td>
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<td>Ab, 4X, Crosstide Assay</td>
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<td>Tracer, 10X, Crosstide Assay</td>
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<tr>
<td>Competitor, Crosstide Assay, 5 µM</td>
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<tr>
<td>FP Dilution Buffer</td>
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<tr>
<td>Kinase Quench Buffer</td>
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</tbody>
</table>

Kit Components

- **P2884**
  - Ab, 4X, Crosstide Assay
  - Tracer, 10X, Crosstide Assay
  - Competitor, Crosstide Assay, 5 µM
  - FP Dilution Buffer
  - Kinase Quench Buffer

- **P2885**
  - Ab, 4X, Crosstide Assay
  - Tracer, 10X, Crosstide Assay
  - Competitor, Crosstide Assay, 5 µM
  - FP Dilution Buffer
  - Kinase Quench Buffer

**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 IC50 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 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.

**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 IC50 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**
Protein Kinase C Assay Kits, Green

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<td>100 assays</td>
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<td>Protein Kinase C Assay Kit, Green</td>
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<tr>
<td>Protein Kinase C Substrate, 100 µM</td>
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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 IC₅₀ values
- Screening of inhibitors of PKC isoforms, PKA, and GSK 3β

Features
- **Fluorescence polarization-based**: Assay is rapid, homogeneous, and performed in solution.
- **Flexible**: Formatted for use with single tubes, or multiwell plates.
- **Versatile**: 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, GSK3β, 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 fluorescein-labeled 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).

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<tr>
<td>Anti-Phosphoserine Antibody, 4X</td>
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<td>PKC Tracer, 50X</td>
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<td>PKC Competitor</td>
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<tr>
<td>PKC Standard Curve Dilution Buffer</td>
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</tr>
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<td>PKC Quench Buffer, 4X</td>
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<td>2.5 mL</td>
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<td><strong>P2748</strong></td>
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<td>PKC Tracer, 50X</td>
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<td>100 µL</td>
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<tr>
<td>PKC Standard Curve Dilution Buffer</td>
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<tr>
<td>PKC Quench Buffer, 4X</td>
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<td>25 mL</td>
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</tbody>
</table>

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 IC₅₀ 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
The following PKC isoforms, available from PanVera®, have been successfully tested with this kit: PKCa (alpha), PKCbI (beta I), PKCbII (beta II), PKCd (gamma), PKCe (delta), PKCε (epsilon), PKCζ (zeta), PKCη (eta) and PKCθ (theta).

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 which contain the R-X-X-S/T consensus motif or the motif R-X-S/T may also have measurable activity in this assay.

All reaction components were added to the diluted enzyme on the plate. Final reaction conditions were: 20 mM Hepes (pH 7.4), 10 mM MgCl₂, 0.1 mM CaCl₂, 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 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 MgCl₂, 0.1 mM CaCl₂, 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.

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

<table>
<thead>
<tr>
<th>Product</th>
<th>Product No.</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Serine Kinase Assay Kit, IκB-α pSer 32</td>
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<td>100 assays</td>
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<td>Serine Kinase Assay Kit, IκB-α pSer 32</td>
<td>P2828</td>
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<tr>
<td>Serine Kinase Assay Kit, IκB-α pSer 36</td>
<td>P2849</td>
<td>100 assays</td>
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<td>Serine Kinase Assay Kit, IκB-α pSer 36</td>
<td>P2850</td>
<td>1,000 assays</td>
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<tr>
<td>IκB-α Peptide, 100 µM, non-phosphorylated</td>
<td>P2826</td>
<td>1 mL</td>
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</table>

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 1 hour, then read on the TECAN Polarion in a 96-well black, round bottom DYNEX plate. The IC₅₀ 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 IκB-α pSer 36 Dilution Buffer. Ten microliters of Anti-IκB-α pSer 36 Antibody, 10X and 10 µL of IκB-α pSer 36 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 1 hour, then read on a TECAN Polarion FP plate reader (Research Triangle Park, NC). The IC₅₀ for the IκB-α pSer 36 competitor was 12 nM (n = 4).
CDK Assay Kits, RbING

<table>
<thead>
<tr>
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<th>Quantity</th>
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<tr>
<td>CDK Assay Kit, RbING</td>
<td>P2928</td>
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<tr>
<td>CDK Assay Kit, RbING</td>
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<td>1,000 assays</td>
</tr>
<tr>
<td>RBING Substrate, 10 µM</td>
<td>P2939</td>
<td>1 mL.</td>
</tr>
</tbody>
</table>

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 IC50 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 G1 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, RbING 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 (RbING) using fluorescence polarization (FP) as the detection method. The CDK Assay Kits, RbING 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 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

**P2928**
- Ab, 4X, CDK Assay, RbING: 2.5 mL.
- RbING Tracer, 10X: 1 mL.
- FP Dilution Buffer: 500 µL.
- Kinase Quench Buffer: 1 mL.

**P2929**
- Ab, 4X, CDK Assay, RbING: 25 mL.
- RbING Tracer, 10X: 10 mL.
- FP Dilution Buffer: 50 mL.
- Kinase Quench Buffer: 10 mL.

Competition Curve Using PanVera®’s Cyclin Dependent Kinase Assay Kit, RbING. The RbING 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-RbING Antibody, 10 µL of 10X RbING 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 IC50 for the RbING competitor was 2 nM (n = 4). The Z' Factor for this assay is 0.7.

References
Threonine Kinase Assay Kits, PDK1

**Applications**
- Ideal for signal transduction research and drug discovery
- Nonradioactive detection of serine/threonine kinases
- High-throughput screening
- Quantitative measurement of inhibitor IC₅₀ 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 IC₅₀ for the PDK1 competitor was 9 nM (n = 4).

**References**
Proteins for Fluorescence Polarization-based Assay Kits

### RECEPTORS

<table>
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<tr>
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<th>Expression System</th>
<th>Part No.</th>
<th>Quantity</th>
<th>Related Products</th>
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</thead>
<tbody>
<tr>
<td>Androgen Receptor</td>
<td>E. coli</td>
<td>P2719</td>
<td>750 pmol*</td>
<td>Assay Coming Soon.</td>
</tr>
<tr>
<td>Estrogen Receptor-α5</td>
<td>Baculovirus</td>
<td>P2187</td>
<td>750 pmol*</td>
<td>Estrogen Receptor-α5 Competitor Assay Kits (P2614, P2698)</td>
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<tr>
<td>Estrogen Receptor-β5</td>
<td>Baculovirus</td>
<td>P2466</td>
<td>750 pmol*</td>
<td>Estrogen Receptor-β5 Competitor Assay Kits (P2615, P2700)</td>
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<tr>
<td>Estrogen Receptor-B1</td>
<td>Baculovirus</td>
<td>P2718</td>
<td>750 pmol*</td>
<td>Estrogen Receptor-B1 Competitor Assay Kits (P2615, P2700)</td>
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<tr>
<td>Glucocorticoid Receptor</td>
<td>Baculovirus</td>
<td>P2812</td>
<td>100 pmol*</td>
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<tr>
<td>Glucocorticoid Receptor</td>
<td>Baculovirus</td>
<td>P2835</td>
<td>100 pmol*</td>
<td>Glucocorticoid Receptor Competitor Assay Kit, Red (P2938)</td>
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†Histidine-tagged  
§GST-fusion

Please inquire for bulk purchases of these proteins.

### KINASES

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<th>Part No.</th>
<th>Quantity</th>
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<td>Recombinant Human</td>
<td>E. coli</td>
<td>P2912</td>
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<tr>
<td>Cyclic AMP-dependent Protein Kinase†</td>
<td>Catalytic Subunit, Recombinant Human</td>
<td>A431 Cells</td>
<td>P2628</td>
<td>10 U</td>
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<tr>
<td>Epidermal Growth Factor (EGF) Receptor</td>
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<td>Baculovirus</td>
<td>P2908</td>
<td>10 µg</td>
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<td>Lyn B1</td>
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<tr>
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<td>Baculovirus</td>
<td>P2295</td>
<td>5 µg</td>
</tr>
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<td>Baculovirus</td>
<td>P2296</td>
<td>5 µg</td>
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<td>Baculovirus</td>
<td>P2352</td>
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<td>10 µg</td>
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<td>P2782</td>
<td>20 µg</td>
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*Active Receptor  
†Histidine-tagged  
‡GST-fusion

Please inquire for bulk purchases of these proteins.
Beacon® 2000 Fluorescence Polarization Systems

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Description</th>
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<tbody>
<tr>
<td>P2300</td>
<td>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.</td>
</tr>
<tr>
<td>P2302</td>
<td>Beacon® 2000 Fluorescence Polarization System, 220 V/240 V° (See description for P2300)</td>
</tr>
<tr>
<td>P2370</td>
<td>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.</td>
</tr>
<tr>
<td>P2372</td>
<td>Beacon® 2000 Full-Range Fluorescence Polarization System, 220 V/240 V° (See description for P2370)</td>
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<tr>
<td>P2810</td>
<td>Beacon® 2000 Red Range Fluorescence Polarization System, 110V</td>
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<tr>
<td>P2811+</td>
<td>Beacon® 2000 Red Range Fluorescence Polarization System, 220V</td>
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</tbody>
</table>

**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.
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 Instrument Specifications

**Highlights**

| **High Precision:** | Less than 2 mP standard deviation, at 1 nM fluorescein, for 0.5 mL sample. |
| **High Sensitivity:** | Accurate to 10⁻¹⁰ M fluorescein in polarization mode and 10⁻¹⁵ 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. |
| **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 x 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.

Beacon® 2000 Accessory Items

### Sample Chambers

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Size</th>
<th>Minimum Volume</th>
</tr>
</thead>
</table>
P2257 | Standard Volume (uses P2245, below) | 500 µL |
P2258 | Low Volume (uses P2244, below) | 100 µL |

### Disposable Test Tubes

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Size</th>
<th>Minimum Volume</th>
</tr>
</thead>
</table>
P2245 | 10 x 75 mm | 500 µL |
P2244 | 6 x 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

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Description</th>
<th>Quantity</th>
</tr>
</thead>
</table>
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)

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Description</th>
<th>Quantity</th>
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</thead>
</table>
P2581 | Beacon® 2000 High Polarization Standard, 1X | 4 mL |
P2505 | Beacon® 2000 Low Polarization Standard, 1X | 4 mL |
P2506 | Beacon® 2000 BGG/Phosphate Buffer | 15 mL |

**Kit Components**
Beacon® 2000 High Polarization Standard, 1X
Beacon® 2000 Low Polarization Standard, 1X
Beacon® 2000 BGG/Phosphate Buffer
### RED (FP) Standardization Kit

**Product No.**
P2888

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

**Kit Components**
- Red Polarization Standard (RPS) 4 mL
- RPS Buffer 8 mL

### Beacon® 2000 UV Standardization Kit

**Product No.**
P2500

**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.

**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

### Beacon® 2000 trp Repressor Kit

(Not: This kit is included with each Beacon® 2000 System purchase)

**Product No.**
P2202

**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.

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

### Beacon® 2000 (FP) DNase Activity Detection Kit

**Fluorescence polarization-based kit**

**Product No.**
P2012

**No. of Assays**
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.

**Kit Components**
- Beacon® Fluorescin 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

**Beacon® 2000 (FP) RNase Activity Detection Kit**

**Fluorescence polarization-based kit**

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<th>Product No.</th>
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**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.

---

**RNase Activity Detection Kit**

**Fluorescence intensity-based kit**

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<th>Product No.</th>
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**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.

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**Beacon® 2000 (FP) Protease Activity Detection Kit**

Fluorescence polarization-based kit

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</tbody>
</table>

**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 A280 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

<table>
<thead>
<tr>
<th>Product No.</th>
<th>No. of Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2006</td>
<td>200</td>
</tr>
</tbody>
</table>

**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 A280 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 A280 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**

<table>
<thead>
<tr>
<th>Product No.</th>
<th>No. of Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2058</td>
<td>≥36</td>
</tr>
</tbody>
</table>

**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

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**Fluorescein-C6 Amine Labeling Kit**

**Product No.**

<table>
<thead>
<tr>
<th>Product No.</th>
<th>No. of Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2099</td>
<td>≥15</td>
</tr>
</tbody>
</table>

**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**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein-C6 succinimidyl ester (FXS)</td>
<td>3 × 2 mg</td>
</tr>
<tr>
<td>Dimethylsulfoxide (DMSO)</td>
<td>2 × 1 mL</td>
</tr>
<tr>
<td>Coupling Buffer, 10X</td>
<td>1 mL</td>
</tr>
<tr>
<td>Quench Buffer, 10X</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

**Bovine Serum Albumin**

10%, Ultrapure Molecular Biology Grade (nonacytlated)

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2489</td>
<td>25 mL, 10% solution</td>
</tr>
<tr>
<td>P2046</td>
<td>100 mL, 10% solution</td>
</tr>
</tbody>
</table>

**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 abattoirs.

**Bovine Gamma Globulin**

0.5% Ultrapure Molecular Biology Grade (acetylated)

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2045</td>
<td>10 mL</td>
</tr>
<tr>
<td>P2255</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

**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, radiiodination 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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PATENTS

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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FAX: (608) 204-5300
email: techsupport@panvera.com

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