POLARSCREEN™ PROGESTERONE RECEPTOR COMPETITOR ASSAY, FAR RED

invitrogen™

Cat. No. PV4299 **Shipping Condition: Dry Ice**

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1. Kit Contents

The PolarScreen™ Progesterone Receptor Competitor Assay, Far Red, Catalog no. PV4299, contains the following:

Component	Composition	Amount	Storage Temp.	Individual Catalog no.
Fluormone™ PL Far Red	400 nM in 50% methanol/water	200 µl	-20 °C	PV4300
PR-LBD	50 mM tris (pH 8.0), 1 M urea, 500 mM KCl, 1 mM EDTA, 5 mM DTT, 50% glycerol	70 pmol	-80 °C	PV4304
PR Far Red Screening Buffer	Proprietary buffer (pH 7.4)	20 ml	20–30 °C	PV4301
DTT, 1 M	in water	1 ml	-20 °C or -80 °C	P2325

2. Materials Required but Not Supplied

The following materials are required but not supplied in the kit:

- Fluorescence polarization instrument with suitable excitation and emission capabilities (see **Instrument Settings**, page 5).
- Pipetting devices for 5–1000 µl volumes, suitable repeater pipetters, or multi-channel pipetters.

- Black, 384-well assay plates. This assay has been optimized for a 40-µl volume in black polypropylene plates. Other plate types may give satisfactory results as well.
- A potent PR ligand, such as mifepristone or progesterone, to serve as a positive control for competition.

3. Introduction

The progesterone receptor (PR) is a member of the nuclear receptor superfamily that mediates the action of progestins. Invitrogen's PolarScreen™ Progesterone Receptor Competitor Assay, Far Red, is a fluorescence polarization (FP)-based competition assay that provides a sensitive and robust method for high-throughput screening of potential PR ligands. The kit uses the novel, tight-binding fluorescent PR ligand Fluormone™ PL Far Red and a human PR ligand-binding domain (PR-LBD) that is tagged with glutathione-S-transferase (GST) in a homogenous mix-and-read assay format. This kit contains enough reagents for 400 40-µl assays.

3.1. Biology of the Progesterone Receptor

Ligand-dependent activation of chaperone-bound progesterone receptor results in conformational changes in the receptor leading to dissociation of the chaperone proteins, receptor dimerization, recruitment of coactivator proteins, and specific interactions with DNA response elements at target genes. The genetic programs regulated by PR involve many aspects of female reproductive function, including the establishment and maintenance of pregnancy. Ligands for PR are used clinically in hormone replacement therapy and contraception.

3.2. Assay Overview

Using the PolarScreen™ Progesterone Receptor Competitor Assay, Far Red, you add PR-LBD to Fluormone™ PL Far Red (the "tracer") in the presence of a test compound in a microtiter plate, and measure the resulting polarization value of the tracer. The shift in polarization value is used to determine the relative affinity of the test compound for PR-LBD.

If the test compound binds to the receptor, it will prevent the formation of the receptor/tracer complex, and the tracer will be free in solution. When the tracer is free in solution, its rotational mobility is greater than when bound to the receptor, resulting in a low polarization value. If the test compound does not bind to the receptor, it will have no effect on formation of the receptor/tracer complex, and the measured polarization value of the tracer will remain high.

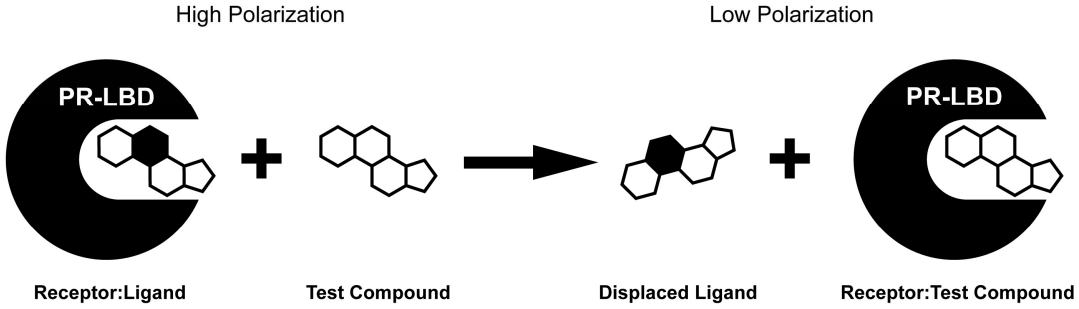

Figure 1. Nuclear Receptor PolarScreen™ Competition Assay Schematic

3.3. Fluorescence Polarization Theory

For detailed information on fluorescence polarization theory and techniques, see the **Fluorescence Polarization Technical Resource Guide** at http://www.invitrogen.com/fpguide.

4. Guidelines and Recommendations

4.1. Receptor/Tracer Binding Affinity

Upon binding to PR-LBD, the fluorescence intensity of Fluormone™ PL Far Red increases. This lowers the observed K_d value for the PR-LBD / Fluormone™ PL Far Red interaction. By applying the method from Lundblad *et al.* (1996), the corrected K_d was calculated to be approximately 9 nM. However, we recommend performing competition experiments using the *observed* EC_{80} concentration of the receptor based on polarization. This concentration has been determined to be approximately 4 nM PR-LBD when using 4 nM Fluormone™ PL Far Red. Sample receptor/tracer binding data is provided in Figure 2.

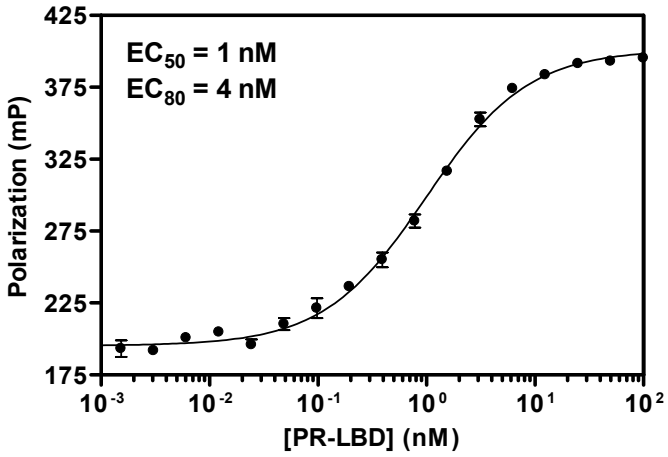

Figure 2. PR-LBD/Fluormone™ PL Far Red Binding Curve. Data points represent the mean polarization value of 4 nM Fluormone™ PL Far Red (±1 standard deviation, n=3) at the indicated concentration of PR-LBD.

4.2. Determining the IC_{50}

You can determine the IC_{50} value for a given test compound by sequentially adding Fluormone™ PL Far Red and PR-LBD at final concentrations of 4 nM each to a dilution series of the test compound. See **Assay Pharmacology**, page 6, for examples of IC_{50} curves.

Note that many test compounds have low solubility in aqueous solutions. Be careful when preparing serial dilutions of these compounds in aqueous solutions to prevent precipitation or carry-over on plastic pipette tips.

4.3. Reagent Handling

PR-LBD

Store PR-LBD at -80 °C. For best results, thaw on ice for 30 minutes before use. Keep on ice once thawed and perform all dilutions while on ice. In concentrated stock solutions, PR-LBD is unstable at temperatures >4°C. Never vortex the PR-LBD stock or dilutions. Do not expose this reagent to more than 4 freeze-thaw cycles.

Fluormone™ PL Far Red

Store Fluormone™ PL Far Red at -20 °C. Thaw on ice for 30 minutes prior to use. This reagent is stable for at least 8 freeze-thaw cycles. Because of the hydrophobic nature of Fluormone™ PL Far Red, we recommend preparing dilutions in glass rather than plastic containers.

PR Far Red Screening Buffer

Thaw PR Far Red Screening Buffer and store at room temperature upon receipt.

4.4. Solvent Tolerance

Up to 4% DMSO, 2% methanol, 2% ethanol, or 16% glycerol may be present in the assay without a significant reduction in the assay dynamic range (ΔmP). However, we always recommend including a compound's vehicle solvent in each of the

control conditions. Note that while glycerol may not affect the ΔmP, it may affect polarization values. Figure 3 contains sample data illustrating the effect of different solvents on the assay ΔmP.

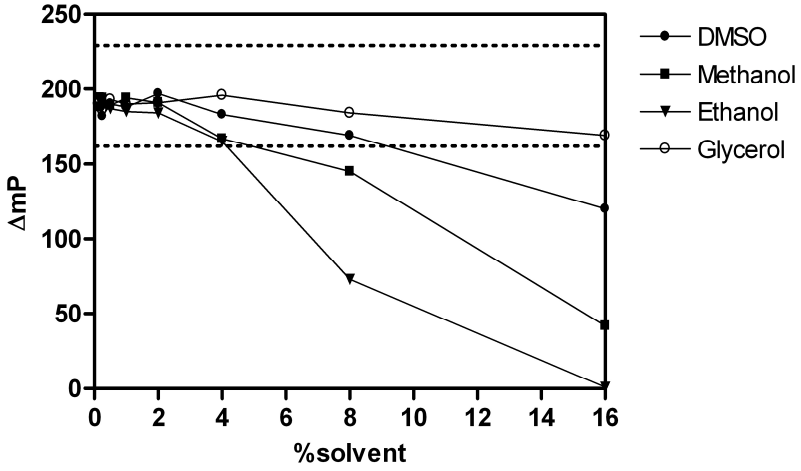

Figure 3. PR Far Red Assay Solvent Tolerance. Dotted lines represent ±3 standard deviations from the mean ΔmP value in the absence of added solvent. ΔmP is determined by the difference in polarization between the receptor/tracer complex and maximum competition by 3 µM mifepristone.

4.5. Note on Reagent Order of Addition

We recommend adding Fluormone™ PL Far Red and PR-LBD separately to the assay plate, to prevent formation of the receptor/tracer complex prior to introduction of the test compound. Pre-formation of the receptor/tracer complex will increase the incubation time required for the assay to reach equilibrium.

Though not recommended, if you want to add a pre-formed receptor/tracer complex to the assay plate, prepare the complex on ice and dispense it to the plate as soon as possible. The resulting assay may require additional time to come to equilibrium, which must be determined by the user.

4.6. Incubation Conditions

The assay has a stable read window of 2–6 hours, where the maximum ΔmP value is stable and excellent Z'-factor values are achieved (see Figure 4).

Incubation Time (hours)	mP	Z'-Factor
2	187	0.73
3	189	0.85
4	183	0.85
6	177	0.83

Figure 4. Effect of Incubation Time on Assay Performance. Sample data represents mean values from 3 separate experiments (n=24). ΔmP was determined by the difference in polarization between the receptor/tracer complex and competition by 1 µM mifepristone. Z'-factor was calculated using the method of Zhang *et al*. (1999) and is an indication of the robustness of the assay. Values > 0.5 are generally considered good, while a value of 1 indicates a theoretically ideal assay with no variability.

4.7. Instrument Settings

The excitation/emission spectra of Fluormone™ PL Far Red are shown in Figure 5. We recommend using excitation/emission wavelengths of 610 nm/670 nm and excitation/emission bandwidths of 20 nm/40 nm (indicated by the shaded bands in the figure). Other filter combinations may perform satisfactorily as well. We recommend using a dedicated dichroic mirror with a reflection cut-off centered between the excitation and emission wavelengths, rather than a generic 50/50 mirror, for optimum sensitivity. This assay was developed using a Tecan Ultra plate reader with the indicated filter set and Cy5 dichroic mirror (Omega Optical, www.omegafilters.com, filter set XF45).

We recommend allowing the instrument to automatically determine optimal gain settings based on wells that contain fully bound Fluormone™ PL Far Red. In addition to a shift in polarization, the tracer undergoes an increase in fluorescence intensity and fluorescence lifetime upon binding to receptor, and so wells containing receptor-bound tracer will have the greatest intensity. Gain settings determined by this method may then be fixed for subsequent assays performed in the same manner.

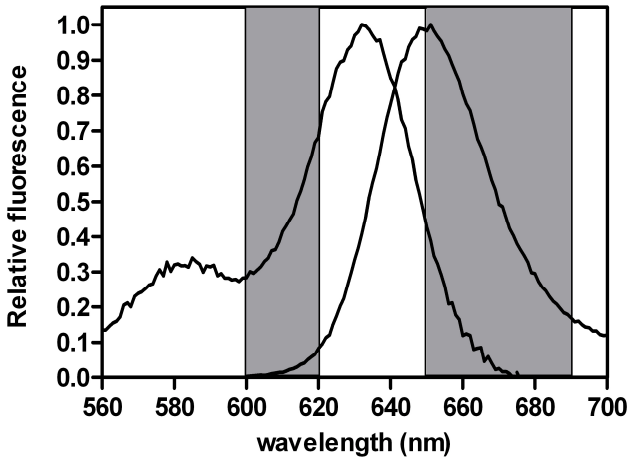

Figure 5. Fluormone™ PL Far Red Excitation/Emission Spectra.

5. Procedure

5.1. Preparing the Reagents

Before proceeding with the assay, prepare the reagents as described in this section.

Complete PR Far Red Screening Buffer

For each milliliter of PR Far Red Screening Buffer, add 2 µl of 1 M DTT and mix thoroughly (2 mM final concentration of DTT). Prepare only enough Complete PR Far Red Screening Buffer for assays to be performed in one day. Prolonged storage of the buffer with DTT results in oxidation of the DTT and subsequent destabilization of the PR-LBD protein. Complete PR Far Red Screening Buffer may be kept at room temperature and used for preparation of all reagents except for the 4X PR-LBD, which should be prepared on ice.

2X Test Compound, Solvent Control, and Control Competitor

Dilute test compound to a 2X concentration in Complete PR Far Red Screening Buffer and mix well. Also prepare a solvent control containing an equivalent amount of the test compound's vehicle solvent in buffer. Include this solvent control as part of the Negative Control. For positive control of competition, prepare a 2X solution of a known PR ligand (we recommend 1 µM mifepristone or 30 µM progesterone final 1X concentration) in buffer. Keep these solutions at room temperature.

4X Fluormone™ PL Far Red

In a glass container, dilute the 400 nM stock solution of Fluormone™ PL Far Red to 16 nM in Complete PR Far Red Screening Buffer. For example, to prepare 1 ml of 4X Fluormone™ PL Far Red, add 40 µl of the 400 nM stock solution to 960 µl of Complete PR Far Red Screening Buffer. Vortex well. Keep this solution at room temperature.

4X PR-LBD

On ice, dilute the PR-LBD stock solution to 16 nM in Complete PR Far Red Screening Buffer. The concentration of the PR-LBD stock is indicated on the tube and its Certificate of Analysis. ***Never vortex the PR-LBD stock or dilutions***. Mix by pipetting or gentle inversion. Keep this solution on ice until needed for dispensing.

5.2. Reagent Volumes

The following table summarizes the reagent amounts and order of addition for each assay condition.

Note: We recommend adding Fluormone™ PL Far Red and PR-LBD separately to prevent formation of receptor/tracer complex prior to introduction of the test compound/control. See **Note on Reagent Order of Addition** on page 4 for more information.

Assay	Reagent Additions	Purpose
Test Compound	1. 20 µl 2X Test Compound 2. 10 µl 4X Fluormone™ PL Far Red 3. 10 µl 4X PR-LBD	Assess competition by test compound of interest using a single point or dilution series
Positive Control	1. 20 µl 2X Control Competitor 2. 10 µl 4X Fluormone™ PL Far Red 3. 10 µl 4X PR-LBD	Represents 100% competition (minimum mP value) by a known, potent progesterone receptor ligand. We recommend using 1 µM mifepristone or 30 µM progesterone as the Control Competitor.
Negative Control	1. 20 µl 2X Test Compound Solvent Control 2. 10 µl 4X Fluormone™ PL Far Red 3. 10 µl 4X PR-LBD	Represents 0% competition (maximum mP value) and accounts for possible interference from a compound's vehicle solvent
Buffer Blank	1. 40 µl Complete PR Far Red Screening Buffer	Serves as a blank for subtraction of background fluorescence

5.3. Performing the Assay

1. In a microtiter plate, pipet the reagents into each well in the order listed in the table above.
2. Cover the assay plate to protect the reagents from light and evaporation, and incubate at room temperature (20–25 °C) for 2–6 hours (see **Incubation Conditions**, page 4).
3. Measure the polarization of each well using a fluorescence polarization plate reader. See page 5 for instrument guidelines. You may want to take multiple readings during incubation to determine whether competition by the test compound of interest has reached binding equilibrium.

6. Assay Pharmacology

Serial dilutions of various test compounds (1% final DMSO concentration) were prepared in 384-well plates. Fluormone™ PL Far Red and PR-LBD were then added to each sample well. The assay was incubated for two hours at room temperature prior to measuring polarization and calculating IC_{50} values. The resulting data is presented in Figure 6. Error bars represent 1 standard deviation from the mean of n=4 for each data point. Curves were fit using a sigmoidal dose-response equation with varying slope:

$$Y = mP_{100\%} + (mP_{0\%} - mP_{100\%})/[1 + 10^{((LogIC50 - X) \times Hill\ Slope)}]$$

Where: Y = mP, X = Log [inhibitor], $mP_{100\%}$ = 100% competition, and $mP_{0\%}$ = 0% competition

Curve fitting was performed using Prism® software from GraphPad™ Software, Inc.

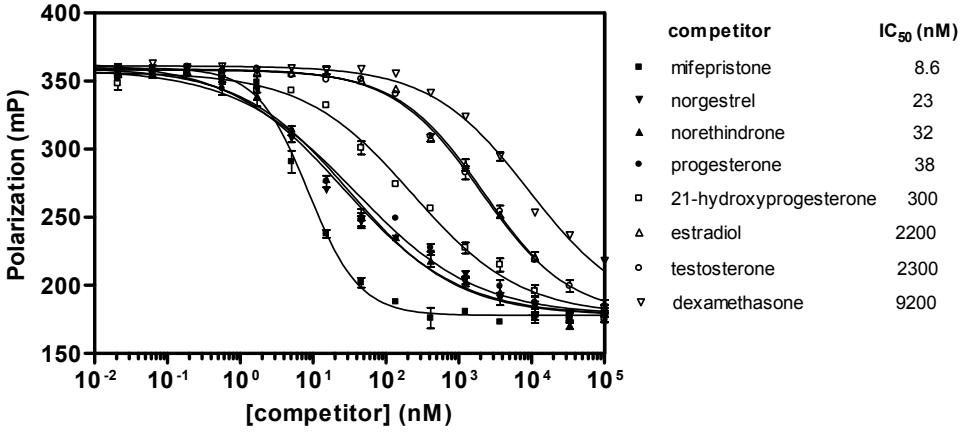

Figure 6. Relative Affinity of Selected Ligands for PR-LBD in the PR Far Red Assay.

7. References

Lundblad, J.R., Laurance, M., and Goodman, R.H. (1996) Fluorescence Polarization Analysis of Protein-DNA and Protein-Protein Interactions. *Molecular Endocrinology 10,* 607–12.

Zhang, Ji-Hu, Chung, T.D.Y., and Oldenburg, K.R. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays, *J. Biomol. Screen 4,* 67–73.