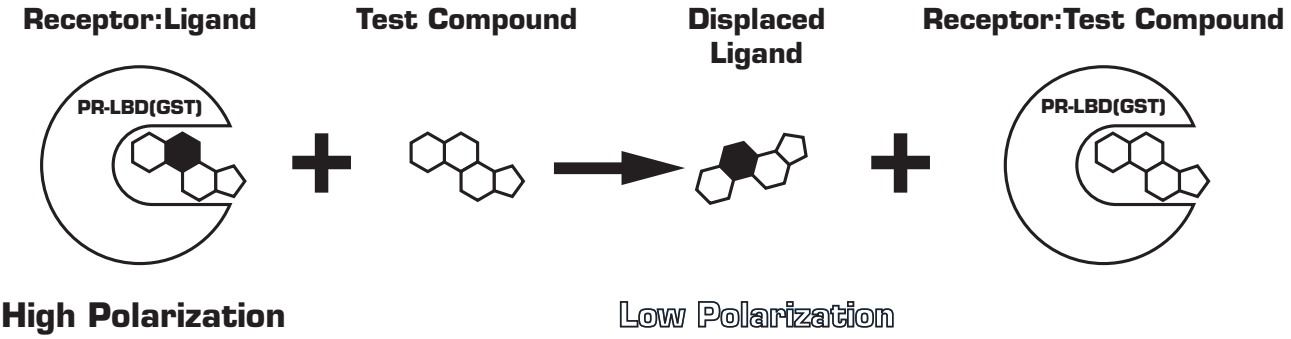
1.0 INTRODUCTION

This kit contains the necessary reagents to perform a competition assay for identification of progesterone receptor binding compounds. An N-terminal fusion of glutathione transferase to the ligand-binding domain of the human progesterone receptor (PR-LBD(GST)) and a proprietary, fluorescently-tagged progesterone ligand (Fluormone™ PL Red) are utilized. The presence of effective competitors prevents the formation of Fluormone™ PL Red/PR-LBD(GST) complex, which has high fluorescence polarization value. The relative binding affinities of test compounds can be determined by measuring the polarization value.



This kit contains enough reagents to perform 100 assays in a total volume of 100 µL in a 96-well plate.

If you would like more information, please see our on-line Fluorescence Polarization Applications Guide at:

http://www.panvera.com/tech/fpguide/index.html

If you would like more information about this specific product, please see our website at:

http://www.panvera.com/catalog/P2962.html

2.0 SAFETY PRECAUTIONS

Normal precautions exercised in handling laboratory reagents should be followed. All reagents in this kit are considered non-hazardous according to 29 CFR 1910.1200. The Fluormone™ PL Red may have steroid activity *in vivo* and therefore should be treated with caution. Methanol is toxic and may be absorbed through the skin. The chemical, physical, and toxicological properties of these products may not, as yet, have been thoroughly investigated. We recommend using gloves, lab coats, eye protection, and a fume hood when working with any chemical reagents.

3.0 DESCRIPTION

3.1 Materials Supplied

Description	Composition	Amount	Part No.
Fluormone™ PL Red	100 nM in 20 mM Tris (pH 7.6), 45% methanol	200 µL	P2964
PR-LBD(GST)	50 mM Tris (pH 8.0), 1M Urea, 500 mM KCl, 1 mM EDTA, 5 mM DTT, 50% glycerol	400 pmol	P2899
Red PR Screening Buffer	Buffer (pH 7.4) containing protein stabilizing agents and glycerol	20 mL	P2966
1 M DTT	in water	1 mL	P2325

3.2 Materials Required but Not Supplied

Multiwell fluorescence polarization instrument with suitable 535 nm excitation and 590 nm emission interference filters
Pipetting devices P20, P200, and P1000, suitable repeater pipetters, or multi-channel pipetters
Black multiwell plates for use in the fluorescence polarization instrument
Red Standardization Kit (PanVera Part No. P2888): recommended for calibrating instrument

3.3 Storage and Stability

Description	Storage Temperature	Notes	Part No.
Fluormone™ PL Red	-20˚C	PR-LBD(GST)/Fluormone™ PL Green complexes should be prepared **in glass** and stored on ice until dispensed.	P2964
PR-LBD(GST)	-80˚C	Avoid repeated freeze-thaw cycles of PR-LBD(GST). **PR–LBD(GST) must remain on ice once thawed.**	P2899
Red PR Screening Buffer	Room Temperature	- -	P2966
1 M DTT	-20˚C	- -	P2325

4.0 ASSAY CONSIDERATIONS

This kit contains enough reagents to perform 100, 100 µL assays in a 96-well plate. A competition curve is generated by adding Fluormone™ PL Red/PR-LBD(GST) complexes to a dilution series of the test compound and then plotting the polarization against the concentration of test compound. The test compound concentration resulting in a half-maximum polarization value shift equals its IC_{50}.

4.1 General Considerations

Controls: Wells that contain each of the following reagents should be included: 1X PR-LBD(GST), 1X Fluormone™ PL Red/PR-LBD(GST) complex, and Red PR Screening Buffer. Also, a control containing Fluormone™ PL Red/PR-LBD(GST) complex plus a competing compound, such as progesterone, may be included.

Note: In the absence of PR-LBD(GST) protein, Fluormone™ PL Red will have a high baseline polarization as its hydrophobic nature causes it to stick to plastic.

Fluormone™ PL Red/PR-LBD(GST) complex preparation: because of the hydrophobic nature of Fluormone™ PL Red, precautionary measures should be taken to minimize its adherence to plastic. We recommend preparing the Fluormone™ PL Red/PR-LBD(GST) complex in **glass**.

Handle PR-LBD(GST) gently: do not vortex. Mix by pipetting.

Solvent tolerances: Up to 0.5% ethanol, 1.25% methanol (in addition to the 0.9% methanol resulting from Fluormone™ PL Red inclusion) and 1.25% DMSO may be added to the assay without a reduction in dynamic range (•mP).

Note: The dynamic ranger will be decreased up to 15 to 20% when 2% methanol or ethanol or 5% DMSO are included in the assay.

Standards: We recommend using 1 nM Red Polarization Standard from the Red Standardization Kit (PanVera Part No. P2888) to determine if the instrument is measuring polarization accurately.

4.2 Competition Experiments

Design the fluorescence polarization competition experiments such that the starting polarization value will represent at least 50% of the maximum shift (*i.e.*, add enough PR-LBD(GST) to bind at least 50% of the Fluormone™ PL Red). The K_d of Fluormone™ PL Red with PR-LBD(GST) is approximately 10 nM. We recommend using 40 nM PR-LBD(GST) to achieve ~80% saturation with a final concentration of 2 nM Fluormone™ PL Red concentration. Fluormone™ PL Red concentrations greater than 2 nM may be required in instruments lacking wavelength-specific dichoric mirrors.

5.0 PROCEDURE

Remove PR-LBD(GST) from the –80˚C freezer and Fluormone™ PL Red from the –20˚C freezer and place on ice for at least 1 hour prior to use.

5.1 Prepare Test Reagents

1. Prepare serial dilutions of the test compounds in Red PR Screening Buffer directly in the multiwell plate. Prepare these dilutions in 50 µL volumes, so that the test compounds will be diluted two-fold when 50 µL PR-LBD(GST)/Fluormone™ PL Red complexes are added in the final reaction.

5.2 Prepare 4 nM Fluormone™ PL Red/PR-LBD(GST) Complex

1. **Calculate the total volume of 2X Complex to be prepared:** 50 µL of 2X Complex is needed per 100 µL assay.
2. **Calculate the volume of each component required:** PanVera recommends 2X Complexes containing 4 nM Fluormone™ PL Red and 80 nM PR-LBD(GST) in Red PR Screening Buffer with 2 mM DTT. In the example shown below, 5 mL of 2X Complex is prepared using a 2000 nM PR-LBD(GST) stock. **Note that the concentration of PR-LBD(GST) is lot-dependent.** Five milliliters of 2X Complex will be sufficient for one 96-well plate. The empty rows of this table may be used as a work sheet for your experiments.

Total complex volume (µL)	[PR-LBD] (nM)	PR-LBD (µL) [80 nM @ 2X]	100 nM Fluormone™ PL Red (µL) [4 nM @ 2X]	1 M DTT (µL) [2 mM DTT @ 2X]	Red PR Screening Buffer (µL)
5000	2000	200	200	10	4590

3. **Prepare the 2X Complex on ice:** Pipet the Red PR Screening Buffer into a **glass tube**. Add the required volume of DTT. Mix PR-LBD(GST) stock gently by pipetting; then add required volume to Red PR Screening Buffer/DTT mixture. Vortex 100 nM Fluormone™ PL Red. Pipette up and down to coat tip, then add needed volume into protein/buffer mixture. Mix well by pipetting.

5.3 Perform the Competition Assay

1. Add 50 µL of 2X Fluormone™ PL Red/PR-LBD(GST) Complex to the multiwell plate wells (already containing 50 µL of the test compound serial dilutions) and mix well by shaking on a plate shaker.
2. Make control wells. A "0% competition" negative control containing 1X Complex, without competitor is recommended. The polarization value for the negative control should be high. A "100% Competition" positive control containing 1X Complex with 10 µM Progesterone is recommended. The polarization value for the "100% Competition" positive control should be low. Fluormone™ PL Red alone **cannot** be used as a "100% Competition" control because its hydrophobic nature causes it to stick to plastic.
3. Cover the plate to protect the reagents from light and incubate for a minimum of 2 hours at room temperature. Fluorescence polarization values can be measured for up to 24 hours.
4. Measure polarization values using a plate reader equipped for measuring fluorescence polarization.

   Note: All wells should be blanked against a control well containing 1X PR-LBD(GST) only. Alternatively, Red PR Screening Buffer can be used as the blank, since the inherent fluorescence of PR-LBD(GST) is very low.

6.0 RESULTS AND DISCUSSION

Below is an example of competition data generated on a 96-well plate. The concentration of test compound resulting in a half-maximum shift in polarization equals the IC_{50} of the compound.

This curve was plotted using the following equation:

$$Y = mP_{100\%} + (mP_{0\%} - mP_{100\%})/1 + 10^{((LogIC50-X)\ \ Hillslope)}$$

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

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