1.0 INTRODUCTION

This kit contains the reagents necessary to perform a competition assay to assess the affinity of test compounds for the human Glucocorticoid Receptor. Glucocorticoid Receptor (GR) is added to a fluorescent glucocorticoid (Fluormone™ GS Red) ligand in the presence of competitor test compounds in microwell plates. The presence of effective competitors prevents the formation of a GS Red/GR complex resulting in a decrease of the polarization value. The shift in polarization value in the presence of test compounds is used to determine relative affinity of test compounds for GR. Additionally, the use of the red-shifted fluorescently labeled glucocorticoid ligand has the added benefit of minimizing background fluorescence interference occasionally found in compound libraries.

Test Compound + GS Red*/GR (high polarization) ↔ Test Compound/GR + GS Red* (low polarization)

This kit is designed for room temperature assays, using a coactivator-related Stabilizing Peptide to maintain GS Red/GR complex integrity (1). Alternatively, the assays may be run in the absence of stabilizing peptide, conducting all procedures at 4˚C, with insignificant change in GR binding characteristics. This kit contains enough reagents to perform 100 assays in 100 µL well volumes.

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

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

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

http://www.panvera.com/catalog/P2893.html

2.0 SAFETY PRECAUTIONS

Normal precautions exercised in handling laboratory reagents should be followed. All reagents in this kit are considered nonhazardous according to 29 CFR 1910.1200. The Fluormone™ GS 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™ GS Red	200 nM in 55% methanol	50 µL	P2894
Glucocorticoid Receptor (GR), Human Recombinant	10% glycerol, 10 mM potassium phosphate (pH 7.4), 10 mM Na_2MoO_4, 0.1 mM EDTA, 5 mM DTT	2 × 25 pmol	P2812
GR Screening Buffer, 10X	100 mM potassium phosphate (pH 7.4), 200 mM Na_2MoO_4, 1 mM EDTA, 20% DMSO	2 × 1 mL	P2814
GR Stabilizing Peptide, 10X	1 mM in 10 mM potassium phosphate (pH 7.4)	2 × 1 mL	P2815
1 M DTT	in water	1 mL	P2325

3.2 Materials Required but Not Supplied

- 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, round-bottom microwell plates for use in the multi-well fluorescence polarization instrument
- Beacon® Red (FP) Standardization Kit (PanVera Part No. P2888), recommended for standardizing fluorescence polarization data
- Dexamethasone, required for the positive control

3.3 Storage and Stability

GR Stabilizing Peptide, 10X (PanVera Part No. P2815), and GR, rHuman (PanVera Part No. P2812) should be stored at –80˚C. **Do not freeze GR on dry ice, as the product is sensitive to pH shifts.** Fluormone™ GS Red (PanVera Part No. P2894) should be stored at –20˚C. DTT (PanVera Part No. P2325) may be stored at either –80˚C or –20˚C. GR Screening Buffer, 10X (PanVera Part No. P2814) may be stored at 20-30˚C. Avoid repeated freeze-thaw cycles of GR (do not expose the reagent to more than 3 freeze-thaw cycles). GR and GS Red/GR complex are unstable at room temperature. GR must remain on ice once thawed.

4.0 GLUCOCORTICOID COMPETITION ASSAY CONSIDERATIONS

This kit contains enough reagents to perform 100 assays in 100 µL well volumes. A competition curve will be generated by adding Fluormone™ GS Red and GR **sequentially** to a dilution series of the test compound. The polarization value will be plotted against the concentration of test compound. The concentration of the test compound that results in a half-maximum shift in polarization value equals the IC_{50} of the test compound.

4.1 General Considerations When Designing a Competition Assay

- **Controls:** A control compound such as cortisol (IC_{50} = 30 ± 10 nM) or dexamethasone (IC_{50} = 10 ± 5 nM) may be included on each plate. In addition, include five control wells that contain one of each of the following: 1X Fluormone™ GS Red, 1X GR, 1X GS Red/GR complex, Complete GR Screening Buffer, and a 1:10 dilution of the Red Polarization Standard (PanVera Part No. P2889, which is found in the Red (FP) Standardization Kit, PanVera Part No. P2888) as your low polarization standard, if necessary.
- **Handle GR gently:** For best results, thaw GR on ice for 1 hour before use. **Never** vortex GR. Keep GR on ice. In the absence of Stabilizing Peptide, GR is unstable in fluorescence polarization experiments at temperatures >8˚C.
- **DMSO and methanol:** The GR competition assay is stable to DMSO and methanol. You may use up to 5% methanol or DMSO in the standard protocol without any loss in dynamic range.
- Once the GS Red/GR Complex has been formed, competition by test compounds is relatively slow. We recommend that GR be added to wells after mixing GS Red and test compounds.
- We recommend using 1 nM Low Polarization Solution and 1X High Polarization Solution from the Beacon® FP One-Step Standardization Kit (PanVera Part No. P2581) with suitable 485 nm excitation and 530 nm emission interference filters to determine if the instrument is measuring polarization values accurately.

4.2 Competition Experiments

Design the fluorescence polarization competition experiments such that the GR/K_d ratio is at least 1, so that the starting polarization value will represent at least 50% of the maximal shift (i.e., add enough GR to bind at least 50% of the Fluormone™ GS Red). The K_d of the Fluormone™ GS Red with GR equals 0.3 ± 0.1 nM. We recommend using 4 nM GR to achieve ~80% saturation with 1 nM Fluormone™ GS Red. Fluormone™ GS Red concentrations greater than 1 nM may be required in polarization instruments lacking wavelength-specific dichroic mirrors.

5.0 PROCEDURE

For experiments to be run in the absence of Stabilizing Peptide, please use the alternate (4˚C) protocol steps in italics.

5.1 Prepare Reagents

1. Remove GR from the –80˚C freezer and thaw on ice for at least 1 hour prior to use.
2. Prepare Complete GR Screening Buffer. Prepare enough buffer for assays to be performed in one day. Prolonged storage of Complete GR Screening Buffer with DTT results in significant yellowing of the buffer over time, which can interfere with fluorescence readings. To prepare buffer for 100 assays, thaw 1 mL of GR Screening Buffer, 10X (PanVera Part No. P2814) and 1 mL of Stabilizing Peptide, 10X (PanVera Part No. P2815) with gentle warming and vortexing; add to 7.95 mL prechilled (4˚C) distilled water with vortexing. Add 50 µL 1 M DTT (PanVera Part No. P2325), vortex, and place on ice.

4˚C Protocol Only: *Prepare Complete GR Screening Buffer in the absence of Stabilizing Peptide. Thaw 1 mL of GR Screening Buffer, 10X (PanVera Part # P2814) with vortexing, and add to 8.95 mL prechilled, distilled water with vortexing. Add 50 µL 1 M DTT, vortex, and place on ice.*

3. Prepare serial dilutions of the test compounds, in Complete GR Screening Buffer, directly in the microwell plate. Prepare these dilutions in 50 µL volumes, so that the test compounds will be diluted two-fold in the final reaction.

5.2 Prepare 4X Fluormone™ GS Red and 4X GR Working Solutions

Prepare enough of the 4X solutions for all reactions being performed. For a total reaction volume of 100 µL, 25 µL of each solution will be required for each well. Fluormone™ GS Red is dissolved in methanol and is therefore quite volatile. Keep this reagent on ice. The recommended concentrations of the 4X solutions are 4 nM Fluormone™ GS Red and 16 nM GR; these concentrations are used in the calculations below.

5.2.1. 4X GS Red Working Solution

1. Calculate the amount of 200 nM Fluormone™ GS Red (PanVera Part No. P2894) needed in the 4X GS Red working solution. In the equation below, record [A] the number of wells needed and [B] the volume of 4X GS Red needed per well. Calculate [C] the volume of stock GS Red needed in microliters. For example, if you need 100 wells, with 50 µL of 4X GS Red per well, include 25 µL of 200 nM Fluormone™ GS Red in the 4X GS Red solution. Add Complete GR Screening Buffer to the final volume ([A] × [B]). Protect 4X Fluormone™ GS Red from light.

   [A]_____ × [B]_____ µL × 0.004 pmol GS Red/µL (4X) ÷ 0.2 pmol GS Red/µL = [C]_____ µL of GS Red needed

   Use the table below for making 4X GS Red. Use the empty rows in the table as a worksheet for your own experiments.

Number of wells [A] (total volume/reaction)	4X GS Red solution in each well [B]	Fluormone™ GS Red [C]	GR Screening Buffer ([A] × [B]) – [C]	Final volume [A] × [B]
100 (100 µL)	25 µL	50 µL	2.45 mL	2.5 mL

5.2.2 4X GR Working Solution

1. Calculate the amount of rHuman GR (PanVera Part No. P2812) needed in the 4X GR. In the equation below, record the number of wells needed [A], the volume of 4X GR needed for each well [B], the desired GR concentration (we recommend 0.016 pmol/µL) [D] and the functional concentration of rHuman GR [E] (taken from the Certificate of Analysis). Calculate the volume of rHuman GR (PanVera Part No. P2812) needed [F], in microliters. For example, if you need 100 wells, with 25 µL of GR per well, and the concentration of GR, rHuman is 0.050 pmol/µL, include 800 µL of GR, rHuman in the 4X GR. **Perform all manipulations of GR on ice.** Make up the rest of the volume with Complete GR Screening Buffer. Keep GR on ice.

   [A]_____ × [B]_____ µL × [D]_____ pmol/mL ÷ [E]_____ pmol/mL = [F]_____ µL of GR needed

   The table below is a recipe for making 4X GR assuming the GR, rHuman (PanVera Part No. P2812) concentration is 0.050 pmol/µL [E]. Use the empty rows in the table as a worksheet for your own experiments.

Number of wells [A] (total volume/reaction)	4X GR in each well [B]	GR [F]	GR Screening Buffer ([A] × [B]) – [F]	Final volume [A] × [B]
100 (100 µL)	25 µL	800 µL	1.7 mL	2.5 mL

5.3 Perform the Competition Assay

***4°C Protocol only**: Perform all steps on ice or at 4°C.*

1. Add 25 µL of 4X GS Red to the microwell plate wells (already containing 50 µL of the serial dilutions of test compounds) and mix well by shaking on a plate shaker.
2. Add 25 µL of 4X GR to the microwell plate wells and mix well by shaking on a plate shaker.
3. Include positive and negative control wells. The negative control should contain 50 µL Complete GR Screening Buffer, 25 µL 4X GS Red and 25 µL 4X GR. This well is used to determine the polarization value with no competitor present, and represents 0% competition. The positive control is identical to the negative control, but includes 1 mM dexamethasone. This well represents 100% competition. Note that the fluorescence polarization value of the positive control will be slightly above background due to nonspecific binding.

4. All wells should be blanked against a control well containing GR only. The GR concentration should be equal in all wells (typically 4 nM). This control corrects for the inherent fluorescence background of the GR preparation.
5. Incubate the plate in the dark at room temperature for 2-4 hours. Fluorescence polarization values are found to vary less than 10% from maximum values if read within this time period.

4˚C Protocol only: *Incubate the plate in the dark at 4˚C for at least 4 hours. FP values are stable for at least 16 hours at 4˚C.*

6. Measure polarization values in each well.

6.0 RESULTS AND DISCUSSION

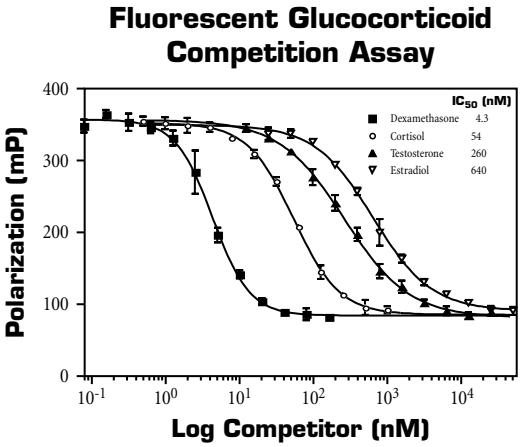
Below is an example of competition data generated on a 96-well plate. The concentration of the test compound that results in a half-maximum shift in polarization value equals the IC_{50} of the test compound, which is a measure of the relative affinity of the test compound for GR. Error bars represent 1 standard deviation from the mean of 10 plate reads.

This curve was plotted using the following equation:

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

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

Curve fitting was performed using Prism® software from GraphPad.



7.0 REFERENCES

1. Chang, C.Y. *et al.* (1999) *Mol. Cell. Biol.* **19:**8226-39.