PolarScreen™ Glucocorticoid Receptor Competitor Assay, Green

Catalog no. P2816 **Storage: See page 2**

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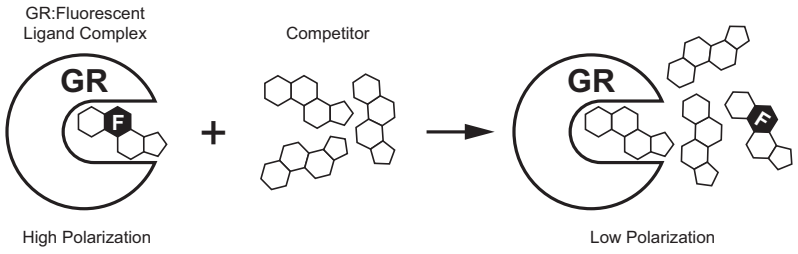
1. Introduction

The PolarScreen™ Glucocorticoid Receptor Competitor Assay, Green kit provides a sensitive and efficient method for high-throughput, fluorescence polarization-based screening of potential glucocorticoid receptor (GR) ligands. The kit uses an insect cell-expressed, full-length, untagged, human glucocorticoid receptor and a novel, tight-binding, fluorescent ligand (Fluormone™ GS1) in a homogenous mix-and-read assay format.

This kit is designed for room temperature assays, using a coactivator-related stabilizing peptide to maintain GS1/GR complex integrity (1). Alternatively, the assay may be run in the absence of the stabilizing peptide, conducting all procedures at 4°C, with an insignificant change in GR binding characteristics. This kit contains enough reagents to perform 100 assays in 100-µL well volumes.

2. Assay Theory



GR is added to a fluorescent glucocorticoid ligand, Fluormone™ GS1, in the presence of individual test compounds in multiwell plates. If a test compound **does not** compete with Fluormone™ GS1 for binding to the GR, then the GR/Fluormone™ GS1 complex will form and Fluormone™ GS1 will tumble slowly during its fluorescence lifetime, resulting in a high polarization value. If a test compound **does** compete with Fluormone™ GS1, the GR/Fluormone™ GS1 complex will not form and Fluormone™ GS1 will tumble rapidly, resulting in a low polarization value. The change in polarization value is used to determine the relative affinity of the test compound for GR.

3. Kit Description

3.1 Materials Supplied

This kit contains enough reagents to perform 100 assays in 100 µL volumes.

Description	Composition	Size	Part #
Fluormone™ GS1	500 nM in 20 mM Tris, 90% methanol (see **Note** below)	50 µL	P2813
Glucocorticoid Receptor (GR), Human Recombinant	Full length human GR is stored in 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
* The concentration of GR is provided on the Certificate of Analysis, included with the product.			

Note on the Concentration of Fluormone

As of November 2009, we have updated our method for calculating the concentration of fluormone. We had been using a method of fluorescent intensity to ensure that FP instruments would be detecting 1 nM of fluormone with uniform intensity lot to lot. We have changed our method to absorbance, as this gives a much more accurate determination of the true concentration of fluormone in solution.

We have **not** changed the concentration of fluormone used in this kit. But we have determined that the actual concentration as determined by absorbance is different than what was determined using fluorescent intensity. To be as clear and as accurate as possible, we are therefore updating the listed concentrations to the values as determined by absorbance. You will notice that the final volumes used in your assays are not affected since the concentration of the reagent and the recommended concentration for the assay have both been updated.

3.2 Materials Required but Not Supplied

- Fluorescence polarization instrument with suitable 485 nm excitation and 535 nm emission interference filters
- Pipetting devices P20, P200, and P1000, suitable repeater pipettors, or multi-channel pipettors
- Black, round-bottom microwell plates for use in the multi-well fluorescence polarization instrument
- FP One-Step Reference Kit (Invitrogen catalog no. P3088). This kit is recommended for validating instrument performance.
- Dexamethasone, required for the positive control

3.3 Storage and Stability

Description	Storage Temperature	Notes
Fluormone™ GS1	–80°C	Avoid repeated freeze/thaws.
Glucocorticoid Receptor (GR), Human Recombinant	–80°C	**Do not freeze on dry ice, as the product is sensitive to pH shifts**. Avoid repeated freeze-thaw cycles (do not expose the reagent to more than 3 freeze-thaw cycles). **Do not vortex.**
GR Screening Buffer, 10X	20–30°C	Avoid repeated freeze/thaws.
GR Stabilizing Peptide, 10X	–80°C	Avoid repeated freeze/thaws.
1 M DTT	–20°C or –80°C	Avoid repeated freeze/thaws.

4. Glucocorticoid Competition Assay

Adding Fluormone™ GS1 and GR **sequentially** to a dilution series of the test compound will generate a competition curve. 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™ GS1, 1X GR, 1X GS1/GR complex, Complete GR Screening Buffer.
- **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 GS1/GR Complex has been formed, competition by test compounds is relatively slow. We recommend that GR be added to wells after mixing GS1 and test compounds.
- We recommend using 1 nM Low Polarization Solution and 1X High Polarization Solution from the FP One-Step Reference Kit (Invitrogen catalog no. P3088) 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™ GS1). The K_d of the Fluormone™ GS1 with GR equals 0.75 ± 0.25 nM. We recommend using 4 nM GR to achieve ~80% saturation with 2.5 nM Fluormone™ GS1. Fluormone™ GS1 concentrations greater than 2.5 nM may be required in polarization instruments lacking wavelength-specific dichroic mirrors.

5. Procedure

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 at room temperature or 4°C, as follows. 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.

   Room temperature preparation protocol: To prepare buffer for 100 assays, thaw 1 mL of GR Screening Buffer, 10X, and 1 mL of Stabilizing Peptide, 10X, with gentle warming and vortexing; add to 7.95 mL prechilled (4°C) distilled water with vortexing. Add 50 µL 1 M DTT, vortex, and place on ice.

   4°C preparation protocol: Prepare Complete GR Screening Buffer in the absence of Stabilizing Peptide. To prepare buffer for 100 assays, thaw 1 mL of GR Screening Buffer, 10X, 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™ GS1 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™ GS1 is dissolved in methanol and is therefore quite volatile. Keep this reagent on ice. The recommended concentrations of the 4X solutions are 10 nM Fluormone™ GS1 and 16 nM GR; these concentrations are used in the calculations that follow.

1. Calculate the amount of 500 nM Fluormone™ GS1 needed in the 4X GS1 working solution. In the equation below, record [A] the number of wells needed and [B] the volume of 4X GS1 needed per well. Calculate [C] the volume of stock GS1 needed in microliters. For example, if you need 100 wells, with 50 µL of 4X GS1 per well, include 25 µL of 500 nM Fluormone™ GS1 in the 4X GS1 solution. Add Complete GR Screening Buffer to the final volume ([A] × [B]). Protect 4X Fluormone™ GS1 from light.

[A]_____ × [B] _____ µL × 0.01 pmol GS1/µL (4X) ÷ 0.5 GS1/µL = [C] _____ µL of GS1 needed

The following table contains the recipe for making 4X GS1. Use the empty rows in the table as a worksheet for your own experiments.

Number of wells [A] (total volume/reaction)	4X GS1 solution in each well [B]	Fluormone™ GS1 [C]	GR Screening Buffer ([A] × [B]) – [C]	Final volume [A] × [B]
100 (100 µL)	25 µL	50 µL	2.45 mL	2.5 mL

2. Calculate the amount of rHuman GR 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 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 following table contains the recipe for making 4X GR assuming the GR, rHuman 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

4.3 Perform the Competition Assay

1. Add 25 µL of 4X GS1 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 GS1 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, or incubate in the dark at 4°C for at least 4 hours. (FP values are stable for at least 16 hours at 4°C.) Fluorescence polarization values are found to vary less than 10% from maximum values if read within this time period.
6. Measure polarization values in each well.

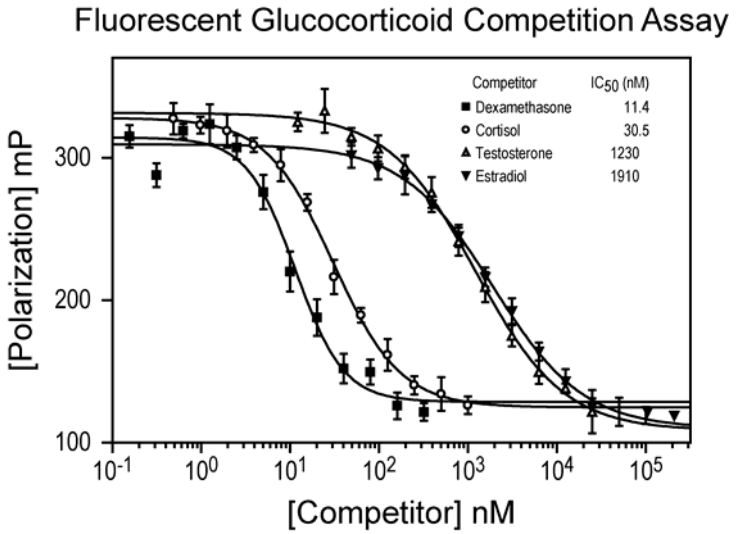
6. Results and Discussion

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:

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

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

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



7. References

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