PPARγ Ligand Screening Assay Kit

Item No. 10007685

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Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. After opening kit, store individual components as stated below.

Item Number	Item	384 wells Quantity	Storage
600065	PPARγ FP Assay Fluorescent Probe - Green	1 vial	-20°C
600066	PPARγ (human recombinant) FP Assay Reagent	1 vial	-80°C
600067	PPARγ FP Assay Ligand Control	1 vial	-20°C
600028	FP Assay Buffer Concentrate (4X)	1 vial	-20°C
10005371	384-Well Solid Plate (black; non-binding)	1 plate	RT
400023	Foil Plate Cover	1 cover	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's PPARγ Ligand Screening Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

E-Mail: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section, on page 3, and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to measure fluorescence polarization using fluorescein as the fluorophore
2. Adjustable pipettes and a multichannel or repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable
4. Microcentrifuge tubes (2 ml)

INTRODUCTION

Background

Peroxisome proliferator-activated receptors (PPARs) are ligand activated nuclear receptors. Three PPAR subtypes have been identified: α, β (also called δ and NUC1), and γ. PPARγ is the most widely studied PPAR and exists in two protein isoforms (γ1 and γ2) due to use of an alternative promoter and alternative splicing.[1] PPARγ is primarily expressed in adipose tissue and to a lesser extent in the colon, immune system, and retina.[2] PPARγ was first identified as a regulator of adipogenesis, but also plays an important role in cellular differentiation, insulin sensitization, atherosclerosis, and cancer. Ligands for PPARγ include fatty acids, arachidonic acid metabolites such as 15-deoxy-$D^{12,14}$-PGJ_2, as well as the thiazolidinedione class of compounds (TZD) which include pioglitazone and rosiglitazone.[3] TZDs are potent, selective PPARγ agonists that lower the hyperglycemia, hyperinsulinemia, and hypertriglyceridemia found in type 2 diabetic subjects and are presently used as oral antidiabetic drugs.[4,5] The use of these synthetic ligands has increased the understanding of PPARγ's mechanism of activation and subsequent biological effects. By increasing our understanding of PPARγ additional drug candidates may be identified.

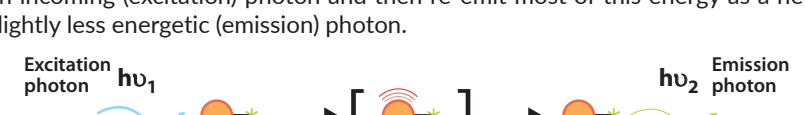
About This Assay

Cayman's PPARγ Ligand Screening Assay Kit provides a convenient fluorescence polarization (FP)-based single step assay for screening PPARγ ligands. In this assay, a ligand of PPARγ was conjugated to fluorescein and is used as the displacement probe. Ligands, agonists, and antagonists of PPARγ will displace the fluorescent probe leading to a decrease in FP. The assay has been validated using known agonists/ligands of PPARγ (Arachidonic Acid, Rosiglitazone, Troglitazone, etc.) with IC_{50} values ranging from nanomolar to millimolar concentrations.

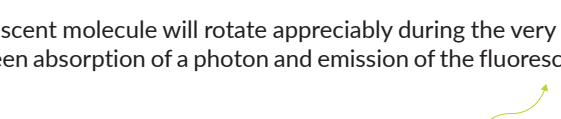
Introduction to FP

Fluorescence polarization (FP) assays are homogeneous, single-step assays ideally suited for high-throughput screening (HTS) of large numbers of samples. All FP assays employ a large molecular species, or binding partner (BP) in conjunction with a small, low molecular weight fluorescent analyte (FA).

Fluorescence is, by definition, the ability of a molecule to absorb the energy of an incoming (excitation) photon and then re-emit most of this energy as a new, slightly less energetic (emission) photon.



A small fluorescent molecule will rotate appreciably during the very small interval of time between absorption of a photon and emission of the fluorescence photon.



If the excitation light is polarized, this rotation will result in complete randomization of the plane of the emitted light. Thus, small fluorescent molecules depolarize an excitation pulse of polarized light (see well #1 in Figure 1, below).

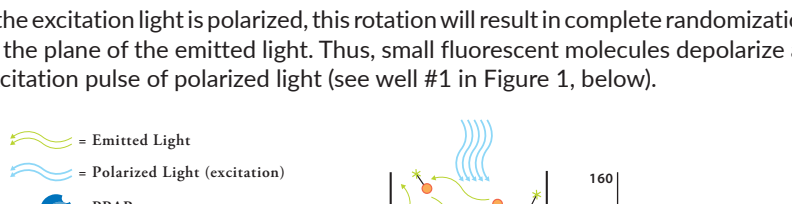


Figure 1.

Large fluorescent molecules do not rotate appreciably in the same small interval of time. They will, therefore, emit light that retains some of the polarization of the polarized excitation light. This polarization is quantified as milli-polarization units, or mP. A fluorescence polarization reader is required to make this measurement.

When a small fluorescent molecule becomes tightly bound to a large one, as in the binding of PPARγ to the fluorescent probe, the rotational speed of the small molecule is abruptly reduced to that of the entire complex as a whole (see well #2 in Figure 2, below).

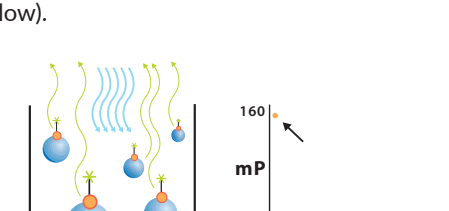


Figure 2.

Therefore, the fluorescent probe bound to PPARγ represents a large fluorescent molecule, which exhibits a high degree of FP. A microplate well containing the fluorescent probe: PPARγ complex will give a high FP reading. The PPARγ Ligand Screening Assay Kit is based on the competition of free ligand in the samples or standards for the high affinity binding site of PPARγ occupied by the fluorescent probe. Addition of a small amount of PPARγ ligand will result in the displacement of the fluorescent probe from the PPARγ binding site (Figure 3, below).

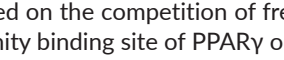


Figure 3.

Some of the fluorescent probe will be released from the PPARγ and will resume its intrinsic, rapid rate of rotation. This will cause a detectable loss of FP in the well (see well #4 in Figure 4, below).

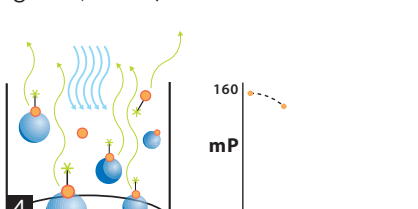


Figure 4.

The addition of large amounts of a PPARγ ligand will result in a much larger reduction in the mP of the well (see well #5 in Figure 5, below). Plotting mP *versus* ligand concentration allows the construction of an IC_{50} curve with a broad dynamic range.

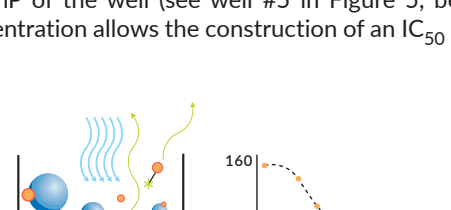


Figure 5.

Cayman's PPARγ Ligand Screening Assay Kit allows for the rapid identification of ligands with a wide range IC_{50} values.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **FP Assay Buffer Concentrate (4X) - (Item No. 600028)**

   This vial contains 6 ml of FP Assay Buffer Concentrate (4X). Prior to use, mix the contents of the vial with 18 ml of deionized water to make a 1X solution. The Assay Buffer may be stored for six months at 4°C. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with deionized water.*

2. **PPARγ FP Assay Fluorescent Probe - Green - (Item No. 600065)**

   This vial contains dried probe. Reconstitute the contents of the vial with 600 µl of diluted FP Assay Buffer.

3. **PPARγ (human recombinant) FP Assay Reagent - (Item No. 600066)**

   This vial contains 350 µl of human recombinant PPARγ. Thaw the enzyme on ice. Once thawed it is ready to use as supplied.

4. **PPARγ FP Assay Ligand Control - (Item No. 600067)**

   This vial contains 60 µl of 5 mM Rosiglitazone. It is ready to use as supplied to prepare the Ligand Control dilutions on page 11.

ASSAY PROTOCOL

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- In a 384-well plate the final volume in the assay is 50 µl in all of the wells. In a 96-well plate the final volume in the assay is 200 µl in all of the wells.
- It is not necessary to use all of the wells on the plate at one time.
- It is recommended to assay the samples in triplicate, but it is the user's discretion to do so.
- The assay is performed at room temperature.
- Monitor the fluorescence polarization with an excitation wavelength of 480-495 nm and an emission wavelength 515-525 nm.

Ligand Control Preparation

Label 8 clean microfuge tubes A-H. Aliquot 468.4 µl DMSO to tube A and 50 µl DMSO to tubes B-H. Transfer 31.6 µl of 5 mM PPARγ FP Assay Ligand Control (Item No. 600067) to tube A and mix thoroughly. Serially dilute by transferring 23.1 µl from tube A to tube B; mix thoroughly. Next, transfer 23.1 µl from tube B to tube C; mix thoroughly. Repeat this process for tubes D-H.

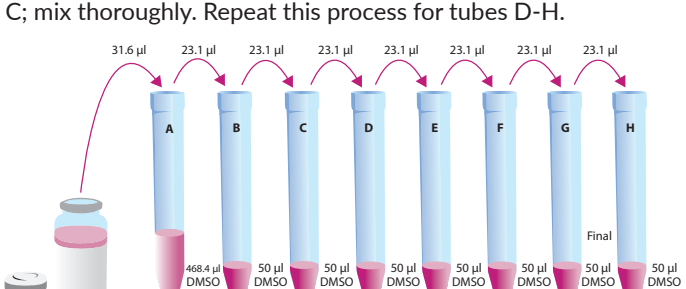


Figure 6. Preparation of the Ligand Control

Performing the Assay

1. **Assay Cocktail**

   Prepare the assay cocktail by mixing the following reagents in a 50 ml conical tube: 18.2 diluted FP Assay Buffer, 500 µl PPARγ FP Assay Fluorescent Probe - Green and 300 µl PPARγ (human recombinant) FP Assay Reagent. *NOTE: 19 ml of Assay Cocktail is enough for either a 384-well, 96-well or higher density plate. Store any unused reagents at -20°C and use within 30 days.*

2. **Maximum Binding Wells**

   Add 47.5 µl of Assay Cocktail and 2.5 µl of 316 µM PPARγ FP Assay Ligand Control to three wells.

3. **Ligand Control Wells**

   Add 47.5 µl of Assay Cocktail and 2.5 µl of ligand control dilution tube B to wells A2 and B2 of a 384-well plate. To wells A3 and B3 add 2.5 µl from tube C. Continue with this procedure until all the standards are aliquoted.

4. **Ligand Sample Wells**

   Add 47.5 µl of Assay Cocktail and 2.5 µl of ligand sample to three wells. *NOTE: Ligands can be dissolved in DMSO, ethanol or methanol. In the event that an appropriate effective displacement is unknown, it is recommended that several dilutions of ligand are assayed.*

5. **Vehicle Wells**

   Add 47.5 µl of Assay Cocktail and 2.5 µl of solvent (same solvent used to dissolve the ligand) to three wells.

6. Cover the plate with the plate cover and incubate for 60-90 minutes at room temperature.

7. Remove the plate cover and read the fluorescence polarization at an excitation wavelength of 480-495 nm and an emission wavelength of 515-525 nm. The fluorescence ploarization signal is stable for at least two hours.

ANALYSIS

Calculations

Fluorescence polarization of a molecule is defined as:

$$\text{Polarization (mP)} = 1{,}000 \times \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + I_{\perp})}$$

A plot of mP *versus* ligand concentration on semi-log axes results in a sigmoidal dose-response curve typical of competitive binding assays. This data can be fit to a 4-parameter logistic equation as shown in Figure 7 (see page 16).

A second method of data analysis uses a logit-log plot. The logit-log method is a transformation based on the following equation:

$$\text{logit}(y) = \ln[y/(1-y)] \qquad \text{where } y = (mP_{standard} \text{ or sample}/mP_{max})$$

The logit transformation reduces the sigmoidal curve of mP *versus* log concentration to a straight line of logit $mP_{standard}/mP_{max}$ *versus* inhibitor concentration on semi-log axes. The curve is completely described by the y-intercept and the slope of the line, which can be used to calculate the concentration values from the logit mP of the samples.

Performance Characteristics

Z' Factor:

Z' factor is a term used to describe the robustness of an assay, which is calculated using the equation below.[6]

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{\left| \mu_{c+} - \mu_{c-} \right|}$$

Where: σ: Standard deviation
μ: Mean
c+: Positive control
c-: Negative control

The theoretical upper limit for the Z' factor is 1.0. A robust assay has a Z' factor >0.5. The Z' factor for Cayman's PPARγ Ligand Screening Assay Kit was determined to be 0.76.

Sample Data

The data shown here is an example of fluorescence polarization data typically produced with this kit; however, your results will not be identical to these. Do not use the data below to directly compare to your samples. Your results could differ substantially.

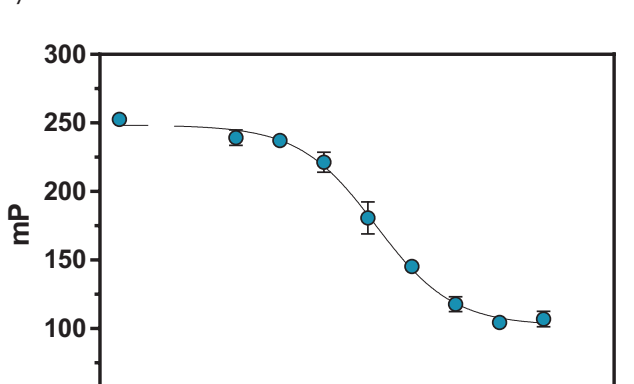


Figure 7. Typical inhibition curves for the displacement of the PPARγ FP Assay Fluorescent Probe - Green by Rosiglitazone. Veh. represents 100% initial activity.

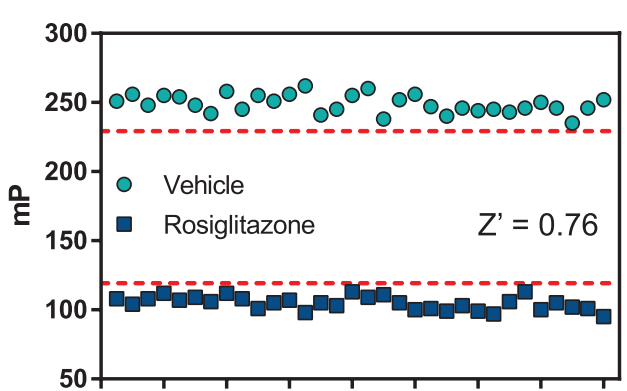


Figure 8. Typical Z' data for the PPARγ Ligand Screening Assay Kit. Data are shown from wells of both positive and negative controls prepared as described in the kit booklet. The calculated Z' factor from this experiment was 0.76. The red lines correspond to three standard deviations from the mean for each control value.

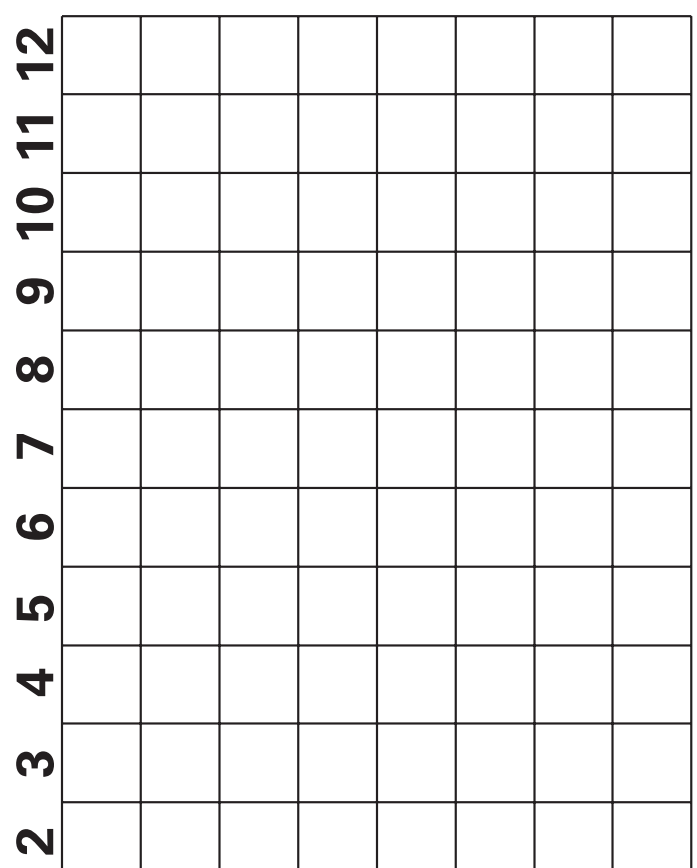
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
A. Erratic values B. Dispersion of duplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells or try more replicates of inhibitor standard to achieve consistency B. Carefully tap the side of the plate with your finger to remove the bubbles
High background mP	Dilution error	Check the dilution of each component

References

1. Vidal-Puig, A., Jimenez-Linan, M., Lowell, B.B., *et al. J. Clin. Invest.* **97**, 2553-2561 (1996).
2. Clark, R.B. *J. Leukoc. Biol.* **71**, 388-400 (2002).
3. Usui, S., Suzuki, T., Hattori, Y., *et al. Bioorg. Medicinal Chem. Letters* **15**, 1547-1551 (2005).
4. Kersten, S., Desvergne, B., and Wahli, W. *Nature* **405**, 421-424 (2000).
5. Sakamoto, J., Kimura, H., Moriyama, S., *et al. Biochem. Biophys. Res. Commun.* **278**, 704-711 (2000).
6. Zhang, J.-H., Chung, T.D.Y., and Oldenburg, K.R. *Journal of Biomolecular Screening* **4(2)**, 67-73 (1999).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.