PPARγ Transcription Factor Assay Kit

Item No. 10006855

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Customer Service 800.364.9897
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GENERAL INFORMATION

Materials Supplied

Kit components may be stored at -20°C prior to use. For long term storage, the Positive Control should be thawed on ice, aliquoted at 25 µl/vial, and stored at -80°C. After opening the kit, we recommend each kit component be stored according to the temperature listed below.

Item No.	Item	Quantity/Size	Storage
10006880	Transcription Factor Binding Assay Buffer (4X)	1 vial/3 ml	4°C
10007472	Transcription Factor Reagent A	1 vial/120 µl	-20°C
10006881	Transcription Factor PPARγ Positive Control	1 vial/150 µl	-80°C
10006882	Transcription Factor Antibody Binding Buffer (10X)	1 vial/3 ml	4°C
10006883	Transcription Factor PPARγ Primary Antibody	1 vial/120 µl	-20°C
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	RT
400035	Polysorbate 20	1 vial/3 ml	RT
10006885	Transcription Factor PPAR Competitor dsDNA	1 vial/120 µl	-20°C
10006884	Transcription Factor Goat Anti-Rabbit HRP Conjugate	1 vial/120 µl	-20°C
10006887	Transcription Factor PPAR 96-Well Strip Plate	1 plate	4°C
400012	96-Well Cover Sheet	1 cover	RT
10006888	Transcription Factor Developing Solution	1 vial/12 ml	4°C
10006889	Transcription Factor Stop Solution	1 vial/12 ml	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.

Kit components may be stored at -20°C prior to use. For long term storage, the Positive Control should be thawed on ice, aliquoted at 25 µl/vial and stored at -80°C. If the assay will be used on multiple days, we recommend each kit component be stored according to the temperatures listed in the booklet.

If You Have Problems

Technical Service Contact Information

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

Fax: 734-971-3641

Email: techserv@caymanchem.com

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 and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 450 nm
2. Adjustable pipettes and a repeating pipettor
3. An orbital microplate shaker
4. A source of UltraPure water; glass Milli-Q or HPLC-grade water are acceptable
6. 300 mM dithiothreitol (DTT)
6. Nuclear Extraction Kit available from Cayman (Item No. 10009277) or buffers for preparation of nuclear extracts

NOTE: The components in each kit lot have been quality assured and warranted in this specific combination only; please do not mix them with components from other lots.

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 the 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-$\Delta^{12,14}$-PGJ_2, as well as thiazolidinediones (TZDs) 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.[4] The use of these synthetic ligands has increased the understanding of PPARγ's mechanism of activation and subsequent biological effects. Modulation of PPARγ by TZDs (pioglitazone and rosiglitazone) are presently used in type 2 diabetes as oral antidiabetic drugs.[5] By increasing our understanding of PPARγ additional drug candidates may be identified.

About This Assay

Cayman's PPARγ Transcription Factor Assay is a non-radioactive, sensitive method for detecting specific transcription factor DNA binding activity in nuclear extracts. A 96 well enzyme-linked immunosorbent assay (ELISA) replaces the cumbersome radioactive electrophoretic mobility shift assay (EMSA). A specific double stranded DNA (dsDNA) sequence containing the peroxisome proliferator response element (PPRE) is immobilized onto the bottom of wells of a 96 well plate (see Figure 1, on page 8). PPARs contained in a nuclear extract bind specifically to the PPRE. PPARγ is detected by addition of specific primary antibody directed against PPARγ. A secondary antibody conjugated to HRP is added to provide a sensitive colorometric readout at 450 nm. The Cayman Chemical PPARγ Transcription Factor Assay detects human, mouse, and rat PPARγ. It will not cross react with PPARδ or PPARα.

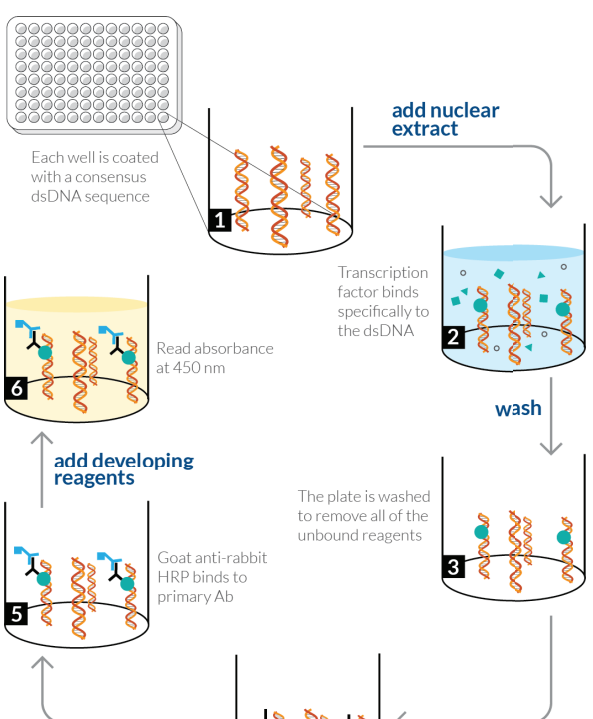


Figure 1. Schematic of the Transcription Factor Binding Assay

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Transcription Factor Antibody Binding Buffer (10X)**

   One vial (Item No. 10006882) contains 3 ml of a 10X stock of Transcription Factor Antibody Binding Buffer (ABB) to be used for diluting the primary and secondary antibodies. To prepare a 1X ABB, dilute 1:10 by adding 27 ml of UltraPure water. Store at 4°C for up to six months.

2. **Wash Buffer Concentrate (400X)**

   One vial (Item No. 400062) contains 5 ml of 400X Wash Buffer. Dilute the contents of the vial to a total volume of 2 liters with UltraPure water and add 1 ml of Polysorbate 20 (Item No. 400035). *NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a pipette. A positive displacement device such as a syringe should be used to deliver small quantities accurately.* A smaller volume of Wash Buffer Concentrate can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Polysorbate 20 (0.5 ml/liter of Wash Buffer). Store at 4°C for up to two months.

3. **Complete Transcription Factor Binding Assay Buffer**

   Prepare 10 ml of Complete Transcription Factor Binding Assay Buffer (CTFB) by adding 2.5 ml of Transcription Factor Binding Buffer (4X) (Item No. 10006880), 0.1 ml of Transcription Factor Reagent A (Item No. 10007472), and 0.1 ml of 300 mM DTT to 7.3 ml of UltraPure water. Scale as necessary. *It is recommended that the CTFB be used the same day it is prepared.*

4. **Transcription Factor PPARγ Positive Control**

One vial (Item No. 10006881) contains 150 µl of clarified cell lysate. This lysate is provided as a positive control for PPARγ activation; it is not intended for plate to plate comparisons. The cell lysate provided is sufficient for 15 reactions and will produce a strong signal (>0.5 AU at 450 nm) when used at 10 µl/well. A decrease in signal may occur with repeated freeze-thaw cycles. It is recommended that the Positive Control be aliquoted at 25-50 µl per vial and stored at -80°C to avoid loss in signal from repeated freeze-thaw cycles.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of PPAR Positive Control (PC), Competitor dsDNA (C1), and samples of nuclear extracts (S1-S44) to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 22).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
B	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
C	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43
D	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36	S44	S44
E	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37	NSB	NSB
F	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38	PC	PC
G	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39	Blk	Blk
H	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40	C1	C1

S1-S44 - Sample Wells
NSB - Non-specific Binding Wells
PC - Positive Control Wells
Blk - Blank Wells
C1 - Competitor dsDNA Wells

Figure 2. Sample plate format

Pipetting Hints

- 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

- Plate strips can be used in separated experiments if stored at 4°C properly in the resealable pouch.
- A minimum of two Blk, two NSB, and two PC wells should be included in each assay.
- We recommend using Cayman's Nuclear Extraction Kit (Item No. 10009277) for preparing your samples.

Performing the Assay

Binding of active PPARγ to the consensus sequence

1. Equilibrate the plate and buffers to room temperature prior to opening. Remove the plate from the foil and select the number of strips needed. The 96-well plate supplied with this kit is ready to use.

   NOTE: If you are not using all of the strips at once, place the unused strips back in the plate packet with the desiccant and store at 4°C.

2. Add appropriate amount of reagents listed below to the designated wells as follows:

   Blk - add 100 µl of CTFB to designated wells.

   NSB - add 100 µl of CTFB to designated wells. Do not add samples or Positive Control to these wells.

   Competitor ***(optional)*** - Add 80 µl of CTFB prior to adding 10 µl of Transcription Factor PPAR Competitor dsDNA (Item No. 10006885) to designated wells. Add 10 µl of control cell lysate or sample.

   S1-S40 - Add 90 µl of CTFB followed by 10 µl of sample to designated wells.

   PC - Add 90 µl of CTFB followed by 10 µl of Positive Control serial dilutions to appropriate wells.

3. Use the 96-well cover sheet (Item No. 400012) provided to seal the plate. Incubate overnight at 4°C or one hour at room temperature on an orbital shaker.
4. Empty the wells and wash five times with 200 µl of 1X Wash Buffer. After the final wash, tap the plate on a paper towel to remove any residual Wash Buffer.

Addition of Transcription Factor PPARγ Primary Antibody

5. Dilute the Transcription Factor PPARγ Primary Antibody (Item No. 10006883) 1:100 in 1X ABB. Add 100 µl to each well except the Blk wells.
6. Use the adhesive cover sheet provided to seal the plate.
7. Incubate the plate for one hour at room temperature on an orbital shaker.
8. Empty the wells and wash each well five times with 200 µl of 1X Wash Buffer. After the final wash, tap the plate three to five times on a paper towel to remove any residual Wash Buffer.

Addition of the Transcription Factor Goat Anti-Rabbit HRP Conjugate

9. Dilute the Transcription Factor Goat Anti-Rabbit HRP Conjugate (Item No. 10006884) 1:100 in 1X ABB. Add 100 µl to each well except the Blk wells.
10. Use the adhesive cover provided to seal the plate.
11. Incubate for one hour at room temperature on an orbital shaker.
12. Empty the wells and wash five times with 200 µl of 1X Wash Buffer. After the final wash, tap the plate three to five times on a paper towel to remove any residual Wash Buffer.

Develop and Read the Plate:

13. Add 100 µl of Transcription Factor Developing Solution (Item No. 10006888) to each well.
14. Incubate the plate for 30 minutes at room temperature on an orbital shaker protected from light. Allow the wells to turn medium to dark blue prior to adding Transcription Factor Stop Solution (Item No. 10006889).
15. Add 100 µl of Stop Solution per well. The solution within the wells will change from blue to yellow after adding the Stop Solution.
16. Read absorbance at 450 nm within five minutes of adding the Stop Solution.

Assay Procedure Summary

NOTE: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when initially performing the assay.

1. Add reagents to wells as indicated in Table 2.
2. Incubate overnight at 4°C or one hour at room temperature on an orbital shaker.
3. Wash each well five times with 200 µl of 1X Wash Buffer.
4. Add 100 µl of diluted PPARγ Primary Antibody per well (except Blk wells).
5. Incubate one hour at room temperature on an orbital shaker.
6. Wash each well five times with 200 µl of 1X Wash Buffer.
7. Add 100 µl of diluted Secondary Antibody (except Blk wells).
8. Incubate one hour at room temperature on an orbital shaker.
9. Wash each well five times with 200 µl of 1X Wash Buffer.
10. Add 100 µl of Developing Solution per well.
11. Incubate 30 minutes on an orbital shaker.
12. Add 100 µl of Stop Solution per well.
13. Measure the absorbance at 450 nm.

Steps	Reagent	Blk	NSB	PC	Competitor	S1-S40
1. Add reagents	CTFB	100 µl	100 µl	90 µl	80 µl	90 µl
	Competitor dsDNA				10 µl	
	Positive Control			10 µl	10 µl	
	Samples					10 µl
2. Incubate	Cover plate and incubate overnight at 4°C or one hour at RT on an orbital shaker					
3. Wash	Wash all wells five times					
4. Add reagents	Primary Antibody		100 µl	100 µl	100 µl	100 µl
5. Incubate	Cover plate and incubate one hour at RT on an orbital shaker					
6. Wash	Wash all wells five times					
7. Add reagents	Secondary Antibody		100 µl	100 µl	100 µl	100 µl
8. Incubate	Cover plate and incubate one hour at RT on an orbital shaker					
9. Wash	Wash all wells five times					
10. Add reagents	Developer Solution	100 µl	100 µl	100 µl	100 µl	100 µl
11. Incubate	30 minutes					
12. Add reagents	Stop Solution	100 µl	100 µl	100 µl	100 µl	100 µl
13. Read	Read plate at wavelength of 450 nm					

Table 2. Quick Protocol Guide

Performance Characteristics

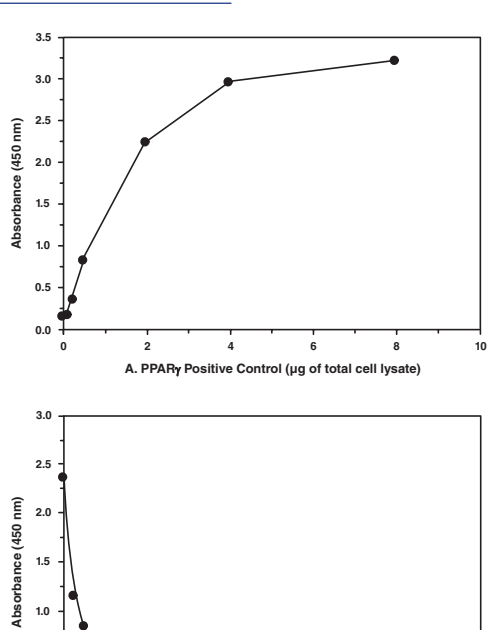


Figure 3. *Panel A:* Increasing amounts of positive control (total lysate) are assayed for PPARγ DNA-binding activity using the Cayman's PPARγ Transcription Factor Assay Kit. *Panel B:* PPARγ DNA-binding assays are performed in the presence of competitive dsDNA. The decrease in signal caused by addition of competitive dsDNA confirms the assay specificity.

Interferences

The following reagents were tested for interference in the assay.

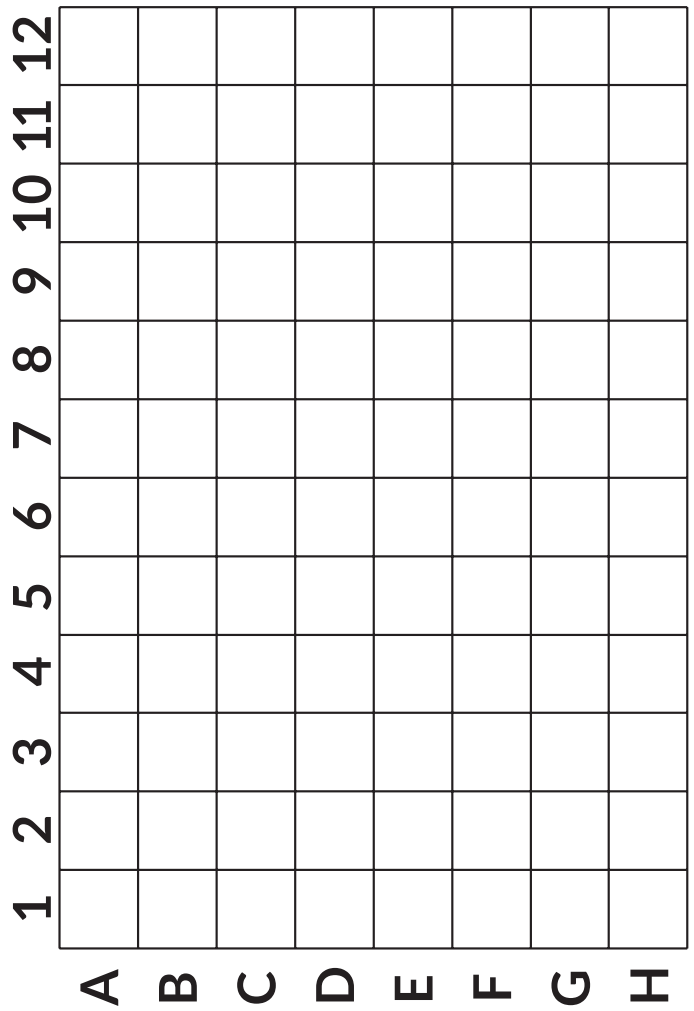
Reagent	Will Interfere (Yes or No)
EGTA (≤1 mM)	No
EDTA (≤0.5 mM)	No
ZnCl (any concentration)	Yes
DTT (between 1 and 5 mM)	No
Dimethylsulfoxide (≤1.5%)	No

Troubleshooting

Problem	Possible Causes	Recommended Solutions
No signal or weak signal in control wells	A. Omission of key reagent B. Plate reader settings not correct C. Reagent expired D. Salt concentrations affected binding between DNA and protein E. Developing reagent used cold F. Developing reagent not added to correct volume	A. Check that all reagents have been added and in the correct order; perform the assay using the Positive Control B. Check wavelength setting on plate reader and change to 450 nm C. Check expiration date D. Reduce the amount of nuclear extract used in the assay, or reduce the amount of salt in the nuclear extracts (alternatively can perform buffer exchange) E. Prewarm the Developing Solution to room temperature prior to use F. Check pipettes to ensure correct amount of Developing Solution was added to wells
High signal in all wells	A. Incorrect dilution of antibody (too high) B. Improper/inadequate washing of wells C. Over-developing	A. Check antibody dilutions and use amounts outlined in instructions B. Follow the protocol for washing wells using the correct number of times and volumes C. Decrease the incubation time when using the developing reagent
High background (NSB)	Incorrect dilution of antibody (too high)	Check antibody dilutions and use amounts outlined in the instructions
Weak signal in sample wells	A. Sample concentration is too low B. Incorrect dilution of antibody C. Salt concentrations affecting binding between DNA and protein	A. Increase the amount of nuclear extract used; loss of signal can occur with multiple freeze/thaw cycles of the sample; prepare fresh nuclear extracts and aliquot as outlined in product insert B. Check antibody dilutions and use amounts outlined in the instructions C. Reduce the amount of nuclear extract used in the assay or reduce the amount of salt in the nuclear extracts (alternatively can perform buffer exchange)

References

1. Vidal-Puig, A., Jimenez-Linan, M., Lowell, B.B., *et al.* Regulation of PPARγ gene expression by nutrition and obesity in rodents. *J. Clin. Invest.* **97**, 2553-2561 (1996).
2. Clark, R.B. The role of PPARs in inflammation and immunity. *J. Leukoc. Biol.* **71**, 388-400 (2002).
3. Usui, S., Suzuki, T., Hattori, Y., *et al.* Design, synthesis, and biological activity of novel PPARγ ligands based on rosiglitazone and 15Δ-PGJ_2. *Bioorg. Medicinal Chem. Letters* **15**, 1547-1551 (2005).
4. Kersten, S., Desvergne, B., and Wahli, W. Roles of PPARs in health and disease. *Nature* **405**, 421-424 (2000).
5. Sakamoto, J., Kimura, H., Moriyama, S., *et al.* Activation of human peroxisome proliferator-activated receptor (PPAR) subtypes by pioglitazone. *Biochem. Biophys. Res. Commun.* **278**, 704-711 (2000).



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.