RayBio® Human PPAR-gamma Transcription Factor Activity Assay

User Manual Version 3
August 17, 2023

Catalog numbers: TFEH-PPARg-1 (1 plate kit)
TFEH-PPARg-2 (2 plate kit)
TFEH-PPARg-5 (5 plate kit)

Please read manual carefully
before starting your experiment.

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3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
Tel: 770-729-2992, Fax:770-206-2393
Web: www.RayBiotech.com, Email: info@raybiotech.com

RayBiotech Life, Inc.

RayBio® Human PPAR-gamma Transcription Factor Activity Assay

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Please read the entire manual carefully before starting your experiment.

I. STORAGE

Upon receipt, the positive control should be removed and stored at or below -20°C. The remainder of the kit can be stored for up to 6 months at 2 to 8°C.

Note: The entire kit can be stored for up to 1 year at -20°C. Avoid repeated freeze-thaw cycles.

II. MATERIALS PROVIDED

COMPONENT	Quantity
PPAR-gamma DNA-coated Microplate*	1 plate
DNA Binding Buffer	4 mL
Positive Control	Lot specific
Specific Competitor Probe	1 vial
Non-Specific Competitor Probe	1 vial
Assay Reagent	200 uL
DTT	200 uL
20X Wash Buffer Concentrate	25 mL
PPAR-gamma Primary Antibody	1 vial
HRP Conjugated Secondary Antibody	1 vial
Antibody Diluent Buffer	25 mL
TMB One-Step Substrate Reagent	12 mL
Stop Solution**	8 mL

*Return unused wells to the pouch containing desiccant pack, reseal along entire edge.
**Contains 0.2 M Sulfuric Acid

III. ADDITIONAL MATERIALS REQUIRED

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Shaker.
3. Pipettes capable of accurately delivering 2 µl to 1 ml volumes.
4. Pipettes capable of delivering 1 – 25 ml volumes for reagent preparation.
5. Graduated cylinders: 100 ml and 1 liter
6. Distilled or deionized water.
7. Tubes to prepare sample dilutions.

IV. REAGENT PREPARATION

NOTE: *Thaw all reagents and buffers to room temperature immediately before use. Keep samples and kit positive control(s) on ice. If wash buffers contain visible crystals, mix gently (at room temperature) until dissolved.*

NOTE: *Briefly centrifuge (~1,000g) all reagent vials before opening to ensure maximum recovery.*

1. Prepare wash buffer, positive control, and antibodies according to following table.

COMPONENT	PREPARATION	STORAGE AFTER PREPARATION
20X Wash Buffer Concentrate	Dilute 20-fold** with distilled or deionized water	1 month at 4°C
Positive Control**	Combine all vials and bring total volume to 200 µl with distilled or deionized water.	Do not store and reuse.
PPAR-gamma Detection Antibody+	Add 100 uL Antibody Diluent Buffer directly to each vial, mix thoroughly, and centrifuge briefly to prepare 100X Antibody Concentrates (Detection and Secondary).	Antibody Concentrate can be stored at 4°C for up to 5 days.
HRP Conjugated Secondary Antibody +	Dilute the 100X Antibody Concentrates 100-fold with Antibody Diluent Buffer to prepare 1X Detection Antibody and 1X HRP Conjugated Secondary Antibody	1X Antibody should be prepared fresh. Do not store and reuse.

*A 20-fold dilution is the same as 1-part 20X Wash Buffer Concentrate and 19-parts water.

**The number positive control vials included with each kit is lot specific. Kits may receive one or more vials.

+100 µl of each reagent is needed per well. Scale up according to number of wells run per assay. We recommend that the calculated volume be multiplied by 1.2 to compensate for pipetting error and to ensure that there is sufficient volume to fill all the wells.

2. Prepare samples: Prepare nuclear or whole cell lysates containing target transcription factor from cell culture or tissue. We recommend using the RayBiotech Nuclear Extraction Kit (Cat # NE-50) to isolate nuclear and cytoplasmic cell fractions for subsequent use in transcription factor activity assays.

3. Prepare transcription factor binding system: Prepare 100 µL transcription factor binding system for each well according to the following table.
Note Reactions can be prepared in a labeled microfuge tube or directly in the coated plate well. If preparing in the coated wells, add reagents in the order they appear in the table (from top to bottom) for best results.

COMPONENT	Positive control	Specific competitor	Non-Specific competitor	Sample	Blank
5x DNA Binding Buffer	20 µl	20 µl	20 µl	20 µl	20 µl
Assay Reagent	1.5 µl	1.5 µl	1.5 µl	1.5 µl	1.5 µl
DTT	1 µl	1 µl	1 µl	1 µl	1 µl
Specific Competitor DNA probe	-	10 µl	-	-	-
Non-specific Competitor DNA probe	-	-	10 µl	-	-
Deionized water	27.5 µl	17.5 µl	17.5 µl	bring total reaction volume to 100µl	77.5 µ
Positive Control*	50 µl	50 µl	50 µl	-	-
Sample	-	-	-	** µl	-

*Use positive control prepared in step 1.

**Amount of sample to be used in this assay must be empirically determined by the investigator.

Note 1:A positive control should be run with each assay to ensure proper kit performance.

Note 2: The specific competitor competes for binding to the transcription factor, whereas the nonspecific probe does not. In this way, these conditions act as controls for transcription factor binding specificity. However, it is not necessary that they be run for individual samples or on every assay.

V. ASSAY PROCEDURE

Note: All incubations and wash steps must be performed at room temperature under gentle rocking or rotation (~1-2 cycles/sec).

1. Bring all reagents to room temperature (18 - 25°C) before use.

2. As prepared above in Section IV, add **100 µl** of each prepared transcription factor binding reaction system (positive control, specific competitor, non-specific competitor, sample(s), and blank) into appropriate wells. Cover wells and incubate for **2 hours.**

   Optional: This incubation step can be run overnight at 4°C.

3. Discard the solution over sink and wash **4 times** with 1x Wash Buffer. Wash by filling each well with **300 µl** 1x Wash Buffer using a multi-channel pipette or auto-washer. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining 1x Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add **100 µl** of prepared 1x Detection Antibody to each well.Incubate for **1 hour**

5. Discard the solution. Repeat the wash as described in Step 3 above.

6. Add **100 µl** of the prepared 1x HRP Conjugated Secondary Antibody to each well. Incubate for **1 hour.**

7. Discard the solution. Repeat the wash as described in Step 3 above.

8. Add **100 µl** of TMB One-Step Substrate Reagent to each well. Incubate for **30 minutes**. Protect from light.

   Note: Proceed immediately to step 9. Do **NOT** wash.

9. Add **50 µl** of Stop Solution directly to each well. Read at 450 nm immediately.

VI. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and controls as instructed.

2. Add 100 µl transcription factor binding reaction system to each well. Incubate for 2 hours at room temperature or overnight at 4°C.

3. Add 100 µl prepared 1X Detection antibody solution to each well. Incubate 1 hour.

4. Add 100 µl prepared 1X HRP-Conjugated Secondary Antibody solution to each well. Incubate 1 hour.

5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes.

6. Add 50 µl Stop Solution directly to each well (do **NOT** wash after TMB step).

7. Read at 450 nm immediately.

VII. TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	SOLUTION
Low signal	Insufficient incubation times	Ensure sufficient incubation time. Assay procedure step 2 may be done overnight at 4°C with gentle shaking.
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation.
	Insufficient transcription factor per well	Check positive control wells and increase the amount of sample.
High CV%	Inaccurate pipetting	Check pipettes.
	Air bubbles in wells	Remove bubbles in wells.
	Cross contamination	Be careful when preparing samples between wells.
High background	Plate is insufficiently washed	Review the manual for proper washing. If using an automated plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer.
	Incorrect antibody dilution(s)	Check antibody dilutions.