KAMIYA BIOMEDICAL COMPANY

Rat Peroxisome Proliferator Activated Receptor Gamma (PPARg) ELISA

For the quantitative determination of rat PPARg in tissue homogenates and other biological fluids

Cat. No. KT-24946

For Research Use Only. Not for use in diagnostic procedures.

Product Information

Rat Peroxisome Proliferator Activated Receptor Gamma (PPARg) ELISA

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INTENDED USE

This ELISA kit is a sandwich enzyme immunoassay for the *in vitro* quantitative measurement of rat PPARg in tissue homogenates and other biological fluids. For research use only. Not for use in diagnostic procedures.

COMPONENTS

Reagents	Quantity
Pre-coated, ready to use 96-well plate	1
Calibrator (lyophilized)	2
Calibrator Diluent	1 × 20 mL
Detection Reagent A	1 × 120 µL
Detection Reagent B	1 × 120 µL
Assay Diluent A (2X concentrate)	1 × 6 mL
Assay Diluent B (2X concentrate)	1 × 6 mL
TMB Substrate	1 × 9 mL
Stop Solution	1 × 6 mL
Wash Buffer (30X concentrate)	1 × 20 mL
Plate sealer for 96 wells	4

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Microplate reader with 450 ± 10 nm filter.
2. Precision single and multi-channel pipettes and disposable tips.
3. Eppendorf Tubes for diluting samples.
4. Deionized or distilled water.
5. Absorbent paper for blotting the microtiter plate.
6. Container for Wash Solution

STORAGE

All reagents should be stored according to their label. The **Calibrators**, **Detection Reagent A, Detection Reagent B** and the **96-well plate** should be stored at -20°C upon receipt. The unused strips should be kept in a sealed bag with the desiccant provided to minimize exposure to damp air. Opened test kits will remain stable until the expiration date, provided they are stored as above.

PRINCIPLE

The microtiter plate provided in this kit has been pre-coated with an antibody specific to PPARg. Calibrators and samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for PPARg. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then the TMB substrate solution is added to each well. Only those wells that contain PPARg, biotin-conjugated antibody and enzyme-conjugated Avidin will

exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulfuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of PPARg in the samples is then determined by comparing the O.D. of the samples to the calibration curve.

SAMPLE COLLECTION AND STORAGE

Tissue Homogenates

The preparation of tissue homogenates will vary depending upon tissue type. For this assay tissues were rinsed in ice-cold PBS (0.02 mol/L, pH 7.0-7.2) to remove excess blood and weighed before homogenization. The tissues were then minced into small pieces and homogenized in 5-10 mL of PBS with a glass homogenizer on ice (a Micro Tissue Grinder works too). The resulting suspension was sonicated with an ultrasonic cell disrupter or subjected to two freeze-thaw cycles to further break the cell membranes. After that, the homogenates were centrifugated for 5 minutes at 5000 x g. Remove the supernatant and assay immediately or aliquot and store at ≤-20℃.

Other Biological Fluids

Centrifuge samples for 20 minutes at 1000 x g. Remove particulates and assay immediately or aliquot samples and store at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

⚠ Note:

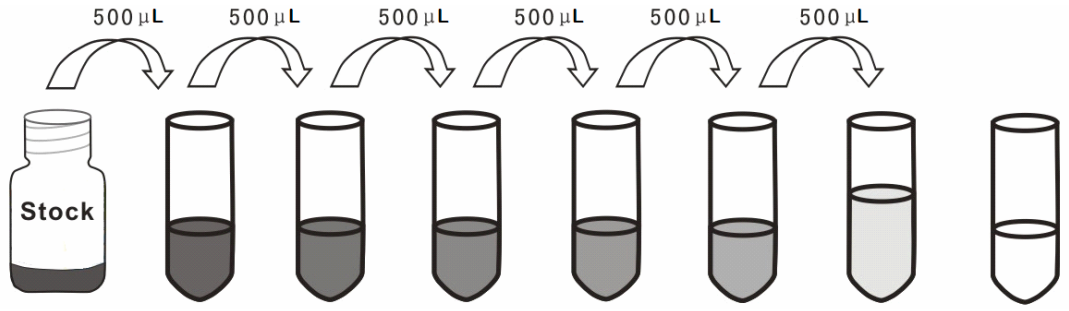
1. Samples to be used within 5 days may be stored at 2-8°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
2. Before performing the assay, slowly bring samples to room temperature.
3. Avoid hemolysis as excessive hemolysis will impact the result.

REAGENT PREPARATION

Bring all kit components and samples to room temperature (18-25°C) before use.

Calibrators

Reconstitute the **Calibrator** with 1.0 mL of **Calibrator Diluent**, kept for 10 minutes at room temperature. Then mix uniformly but gently, avoid foaming. The concentration of the stock calibrator solution is now 10 ng/mL. Use the stock calibrator solution and the **Calibrator Diluent** to produce a dilution series. Pipette 500 µL of **Calibrator Diluent** into each tube. Then perform a serial dilution, beginning with the stock calibrator solution, to create the other calibrators (as shown below). Make sure to use a new pipette tip for each transfer and mix each tube thoroughly before the next transfer. The calibrators will have concentrations of 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 ng/mL, and the last tube filled with only **Calibrator Diluent** will serve as the blank at 0 ng/mL.



Tube	1	2	3	4	5	6	7	8
ng/mL	10	5	2.5	1.25	0.625	0.312	0.156	0

Assay Diluent A and B

Dilute 6 mL of Assay Diluent A or B Concentrate (2X) with 6 mL of deionized or distilled water to prepare 12 mL of Assay Diluent A or B. The prepared working dilution can not be frozen.

Detection Reagent A and B
Briefly spin or centrifuge the stock Detection Reagent A and Detection Reagent B before use. Dilute to the working concentration with working **Assay Diluent A or B,** respectively (1:100).

Wash Solution
Dilute 20 mL of Wash Solution Concentrate (30X) with 580 mL of deionized or distilled water to prepare 600 mL of Wash Solution (1X).

TMB Substrate
Aspirate the needed dosage of the solution with sterilized tips and do not return the residual solution to the vial.

Note:
1. Prepare the calibrators within 15 minutes of beginning the assay. Do not dissolve the reagents at 37°C.
2. Do not perform your serial dilutions directly in the wells.
3. Carefully reconstitute Calibrators or working Detection Reagent A and B according to the instruction. Avoid foaming and mix gently until the crystals have completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to draw more than 10 µl for pipetting.
4. The reconstituted Calibrators, Detection Reagent A and Detection Reagent B can be **used only once**.
5. If crystals have formed in the Wash Solution concentrate (30X), warm to room temperature and mix gently until the crystals have completely dissolved.
6. Distilled water is recommended for the preparation of reagents and samples. Contaminated water or containers for reagent preparation will influence the detection result.

SAMPLE PREPARATION
1. Kamiya Biomedical Company is only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.

2. Please predict the concentration before assaying. If values for these are not within the range of the calibration curve, users must determine the optimal sample dilutions for their particular experiments.

3. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.

4. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals.

5. Owing to the possibility of mismatching between antigen from other resource and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.

6. Influenced by the factors including cell viability, cell number and also sampling time, samples from cell culture supernatant may not be detected by the kit.

7. Fresh samples without long time storage is recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

ASSAY PROCEDURE

Estimate the sample PPARg concentration before assaying. If the estimated values are not within the range of the calibration curve, users must determine the optimal sample dilutions for their particular experiments.

1. Determine wells to be used for diluted calibrators, blank and samples. Prepare 7 wells for calibrators, 1 well for blank. Add 100 µL each of dilutions of calibrators (see Reagent Preparation), blank and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 2 hours at 37°C.

2. Remove the liquid from each well, do not wash.

3. Add 100 µL of the **Detection Reagent A** working solution to each well. Incubate for 1 hour at 37°C after covering with the Plate sealer.

4. Aspirate the solution and wash each well with 350 µL of 1X Wash Solution using a squirt bottle, multi-channel pipette, manifold dispenser or auto-washer, and let sit for 1~2 minutes. Remove the remaining liquid from all wells completely by sharply striking the plate on absorbent paper. Repeat wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

5. Add 100 µL of **Detection Reagent B** working solution to each well. Incubate for 30 minutes at 37°C after covering with the Plate sealer.

6. Repeat the aspiration/wash process five times as in step 4.

7. Add 90 µL of **Substrate Solution** to each well. Cover with a new Plate sealer. Incubate for 15 - 25 minutes at 37°C (Do not exceed 30 minutes). Protect from light. The solution will turn blue after the addition of Substrate Solution.

8. Add 50 µL of **Stop Solution** to each well. The liquid will turn yellow after the addition of Stop solution. Mix the liquid by gently tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.

9. Remove any drops of solution or fingerprints on the bottom of the plate and confirm there are no bubbles on the surface of the liquid. Then, run the microplate reader and conduct measurements at 450 nm immediately.

⚠ Note:

1. **Assay preparation:** Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Unused strips should be resealed and stored at -20°C until the expiration date.
2. **Sample or reagent additions:** Use freshly prepared Calibrators**.** Carefully add samples to wells and mix gently to avoid foaming. Do not touch the well walls if possible. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all calibrators and samples, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of each calibrator level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. **Incubation:** To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. After reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be observed.
4. **Washing:** The wash procedure is critical. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drops of solution or fingerprints on the bottom of the plate. Insufficient washing will result in poor precision and falsely elevated absorbance reading.
5. **Controlling of reaction time:** Observe the change of color after adding **TMB Substrate** (e.g. observation once every 10 minutes)**,** if the color is too dark, add **Stop Solution** in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. **TMB Substrate** is easily contaminated. Protect from light.

CALCULATION OF RESULTS

Average the duplicate readings for each calibrator, control, and sample and subtract the average zero calibrator optical density. Create a calibration curve on log-log graph paper, with PPARg concentration on the y-axis and absorbance on the x-axis. Draw the best fit line straight through the calibration points and it can be determined by regression analysis. Using some plot software is also recommended. If samples have been diluted, the concentration read from the calibration curve must be multiplied by the dilution factor.

PERFORMANCE

Detection Range

The detection range is: 0.156-10 ng/mL.

The calibration curve concentrations used for the ELISA's were 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 ng/mL.

Sensitivity

The minimum detectable dose of Rat PPARg is typically less than 0.049 ng/mL. The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined by the mean O.D. value of 20 replicates of the zero calibrator plus three standard deviations.

Specificity

This assay has high sensitivity and excellent specificity for detection of Rat PPARg. No significant cross-reactivity or interference was observed.

Note: Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between rat PPARg and all the analogues, therefore, cross reaction may still exist.

ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and calibrators;
2. Add 100 µL calibrator or sample to each well. Incubate 2 hours at 37℃;
3. Add 100 µL prepared Detection Reagent A. Incubate 1 hour at 37℃;
4. Aspirate and wash 3 times;
5. Add 100 µL prepared Detection Reagent B. Incubate 30 minutes at 37℃;
6. Aspirate and wash 5 times;
7. Add 90 µL Substrate Solution. Incubate 15-25 minutes at 37℃;
8. Add 50 µL Stop Solution. Read at 450 nm immediately.

IMPORTANT NOTES

1. The final experimental results will be closely related to operation skills of the end users and the experimental environments. Please make sure that sufficient samples are available.
2. Kits from different batches may be a little different in detection range, sensitivity and color developing time. Please perform the experiment exactly according to the instruction attached in kit while electronic ones from our website (www.k-assay.com) is only for information.
3. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
4. Protect all reagents from strong light during storage and incubation. All the bottle caps of reagents should be covered tightly to prevent the evaporation and contamination of microorganism.
5. There may be some foggy substance in the wells when the plate is opened at the first time. It will not have any effect on the final assay results. Do not remove microtiter plate from the storage bag until needed.
6. Wrong operations during the reagents preparation and loading, as well as incorrect parameter setting for the plate reader may lead to incorrect results. A microplate plate reader with a bandwidth of 10 nm or less and an optical density range of 0-3 O.D. or greater at 450 ± 10 nm wavelength is acceptable

for use in absorbance measurement. Please read the instruction carefully and adjust the instrument prior to the experiment.

7. Even the same operator might get different results in two separate experiments. In order to get better reproducible results, the operation of every step in the assay should be controlled. Furthermore, a preliminary experiment before assay for each batch is recommended.
8. Each kit has been strictly passed Q.C. test. However, results from end users might be inconsistent with our in-house data due to some unexpected transportation conditions or different lab equipments. Intra-assay variance among kits from different batches might arise from above factors, too.
9. Kits from different manufacturers for the same item might produce different results, since we haven't compared our products with other manufacturers.
10. The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

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