PPAR-gamma DNA-Binding ELISA Kit

Catalog No.: abx596534

Size: 96T

Range: Qualitative

Sensitivity: Qualitative

Storage: Store the Wild-Type Consensus dsDNA Oligonucleotide, Mutant Consensus dsDNA Oligonucleotide, Binding Buffer (2X), Nuclear Wash Buffer, Cytoplasmic Extraction Buffer and the Nuclear Extraction Buffer at -20°C, and all other components at 4°C for up to 6 months.

Application: For qualitative detection of activated PPAR-gamma transcription factor in nuclear and cell lysates.

Principle of the Assay

This kit is based on indirect enzyme-linked immuno-sorbent assay technology, in combination with an interaction assay. The 96-well plate is pre-coated with streptavidin bound to biotin-conjugated double-stranded oligonucleotides. The controls and samples are added to the wells and incubated. Any transcription factors present in the wells bind to the dsDNA oligonucleotide. The primary antibody specific to PPAR-gamma is added to each well, followed by incubation. HRP-conjugated secondary antibody is used as the detection antibody, which is added to each well, followed by incubation. Unbound conjugates are washed away using wash buffer. TMB substrate is used to visualize HRP activity. TMB is catalysed by HRP to produce a blue colour product that changes to yellow after adding stop solution. The intensity of the yellow colour is proportional to the PPAR-gamma amount bound on the plate. The O.D. absorbance is measured spectrophotometrically at 450 nm in a microplate reader.

Kit components

1. One Pre-coated 96-well Microplate (12 × 8 well strips)
2. Nuclear Lysate Positive Control: 1 vial
3. PPAR-gamma Primary Antibody (100X): 100 µl
4. Primary Antibody Diluent Buffer: 12 ml
5. HRP-Conjugated Anti-Rabbit IgG Antibody: 12 ml
6. Wild-Type Consensus dsDNA Oligonucleotide: 10 µl
7. Mutant Consensus dsDNA Oligonucleotide: 10 µl
8. Cytoplasmic Extraction Buffer: 6 ml
9. Nuclear Extraction Buffer: 6 ml
10. Binding Buffer (2X): 12 ml
11. Wash Buffer (10X): 2 × 25 ml
12. Nuclear Wash Buffer: 12 ml
13. TMB Substrate: 12 ml
14. Stop Solution: 12 ml
15. Plate Sealer: 2

Material Required But Not Provided

1. 37°C incubator
2. Microplate reader
(Wavelength: 450 nm, Correction Wavelength: 540 or 570 nm)
3. Multi and single channel pipettes and sterile pipette tips
4. Squirt bottle or automated microplate washer
5. ELISA orbital shaker
6. Deionised or distilled water
(Recommended TOC: 1-50 ppb; Recommended Resistivity: 18 MΩ · cm)
7. Phenylmethylsulfonyl fluoride (PMSF)
8. Dimethylsulfoxide (DMSO)
9. Protease inhibitor cocktail
10. Phosphate-buffered saline (PBS)
11. 5 M Sodium chloride (NaCl)
12. Glycerol
13. 1-1.5 ml microcentrifuge tubes, and 50 ml tubes
14. 100 ml and 1 L graduated cylinders
15. Absorbent filter papers

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Protocol

A. Preparation of Samples and Reagents

1. Sample Preparation

- This kit is designed for the detection and qualitative analysis of endogenous levels of activated transcription factor PPAR-gamma in a variety of nuclear and cell lysates. Samples should maintain the natural and active form of the target transcription factor. Additional reagents not included in this kit may be required for nuclear extraction from cell culture.
- Tissue homogenates and heterogenous mixtures may contain contaminants which can interfere with the assay. It is recommended to test for interference by testing at least two different dilutions of the sample. If testing demonstrates good correlation between concentration/dilution factor and the OD, purification may not be required. However, if good correlation is not achieved or seen, further purification is recommended. Any visible precipitate or pellet must be clarified prior to use in the assay; it is recommended to centrifuge the sample for 10 minutes at ≥ 10,000 × g.
- It is recommended to carry out several sample dilutions to determine the optimal dilution factor, and to run each dilution in duplicate. Ideal OD readings will fall within the detectable range of the assay, which is dependent on the spectrophotometer used. The end user should determine the optimal dilution factor.
- Samples should be analyzed immediately or aliquoted and stored at 2-8°C if they are to be assayed within 24 hrs. For long-term storage, aliquot and store samples at -80°C. Avoid multiple freeze-thaw cycles.
- Many transcription factors may not be readily expressed in normal cell culture, therefore cell stimulation may be required to increase expression of the target protein.
- Samples that contain NaN_3 cannot be detected as it interferes with HRP.

2. Wash Buffer

Dilute the concentrated wash buffer 10-fold (1/10) with distilled water (e.g. add 20 ml of concentrated wash buffer into 180 ml of distilled water to prepare 200 ml of 1X Wash Buffer).

3. Preparation of PPAR-gamma Primary Antibody Working Solution: prepare immediately prior to starting the assay.

a) Calculate the total volume of 1X PPAR-gamma Antibody required: 100 µl / well × quantity of wells. Allow 50-100 µl more than the total volume.

b) Dilute the 100X PPAR-gamma Antibody with Primary Antibody Diluent Buffer at 1/100 and mix thoroughly (e.g. Add 100 µl of 100X PPAR-gamma antibody into 9900 µl of Primary Antibody Diluent Buffer).

4. Preparation of 1X Working Binding Buffer Solution: only prepare immediately prior to starting the assay.

Prepare the 1X Working Binding Buffer Solution by diluting the concentrated Binding Buffer 2-fold (1/2) with distilled water (e.g. add 60 µl of 2X Binding Buffer into 60 µl of distilled water to prepare 120 µl of 1X Working Binding Buffer Solution).

5. Preparation of Nuclear Lysate Positive Control Solution: keep on ice throughout the assay.

Reconstitute the Nuclear Lysate Positive Control with 110 µl of 1X Working Binding Buffer Solution. The nuclear lysate positive control working solution should be used immediately or aliquoted and stored at -80°C for up to 6 months.

6. Preparation of 100 mM PMSF Stock Solution: only prepare immediately prior to starting cytoplasmic or nuclear extraction.

Dissolve 0.175 g of PMSF in 10 ml of DMSO. Aliquot into 1 ml tubes and store any unused solution at -20°C.

7. Preparation of Protease/Phosphatase Inhibitor (PPI) Buffer Solution: only prepare immediately prior to starting cytoplasmic or nuclear extraction.

Add 250 µl Protease Inhibitor Cocktail to each 5 ml of 1X PBS, then add 100 mM PMSF to the mixture to a final concentration of 1 mM PMSF.

8. Preparation of Working Cytoplasmic Extraction Buffer Solution: only prepare immediately prior to starting cytoplasmic or nuclear extraction.

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Add 250 µl Protease Inhibitor Cocktail to each 5 ml of Cytoplasmic Extraction Buffer, then add 100 mM PMSF to the mixture to a final concentration of 1 mM PMSF.

9. Preparation of Working Nuclear Extraction Buffer Solution: only prepare immediately prior to starting nuclear extraction.
Add 250 µl Protease Inhibitor Cocktail to each 5 ml of Nuclear Extraction Buffer, then add 100 mM PMSF to the mixture to a final concentration of 1 mM PMSF.

B. Nuclear and Cytoplasmic Extraction

This protocol is a recommended procedure for nuclear and cytoplasmic extraction from cultured cells, and can be optimized for specific experiments and applications. Please note that PMSF is unstable and buffers containing PMSF have a shelf life of 24 hours at 4°C.

Cytoplasmic Extraction Procedure:

1. Suspension cells: Collect by centrifuging at 500 × g for 5 minutes at 4°C, then wash once with cold 1X PBS.
   Adherent cells: Wash the cell culture plate twice with cold 1X PBS. Add 0.5 ml of cold PPI Buffer Solution to each plate. Dislodge cells with a cell scraper and collect cells in a pre-chilled 50 ml tube. Wash the plate once more with cold PPI Buffer Solution and collect any remaining cells in the same 50 ml tube. Centrifuge the cell suspension at 500 × g for 5 minutes at 4°C.
2. Resuspend the pellet in (5 × pellet volume) Working Cytoplasmic Extraction Buffer Solution. Transfer the contents to a pre-chilled 2 ml tube and keep on ice for 5 minutes.
3. Centrifuge the tube at 3000 × g for 4 minutes at 4°C. Transfer the supernatant, which contains cytoplasmic lysate, to a new pre-chilled 2 ml tube. The pellet contains nuclear material. If the cytoplasmic lysate is not analyzed immediately, it is recommended to add glycerol to a final concentration of 10%, and then aliquot and store at -80°C.

Nuclear Extraction Procedure:

4. After transferring the cytoplasmic lysate, resuspend the pellet in 1-2 ml of Nuclear Wash Buffer. Centrifuge at 3000 × g for 4 minutes at 4°C. Discard the supernatant, then resuspend the pellet in (2 × pellet volume) Working Nuclear Extraction Buffer Solution. If the change in volume after the second resuspension is ≥ 50 µl, add (0.1 × pellet volume) of 5 M NaCl, then incubate the tube at 4°C for 30 minutes on an orbital shaker.
5. Centrifuge at maximum speed for 10 minutes at 4°C. Collect the supernatant, which is the nuclear extract.
6. Determine the concentration of the nuclear extract using a Bradford Assay or other means.
7. If the nuclear extract is not analyzed immediately, aliquot and store at -80°C. Avoid repeated freeze/thaw cycles.

C. Assay Procedure

1. Set test sample, primary antibody negative control, nuclear lysate positive control, wild-type oligo control (optional), mutant oligo control (optional), and blank wells on the pre-coated plate respectively, and record their positions. It is recommended to measure in duplicate or triplicate.
   • **Sample:** Transcription factors are expressed differently across various tissues, cell types, growth stages, and culture conditions. It is recommended to use at least 5 µg of cell lysate per well, though the optimal amount of sample to test would need to be determined by the end user. If the sample concentrations are unknown, it is recommended to test several dilutions. It is recommended to perform a primary antibody negative control (i.e. add sample without primary antibody) to determine sample background noise. Samples should be diluted in 1X Working Binding Buffer Solution.
   • **Nuclear Lysate Positive Control:** Dilute the Nuclear Lysate Positive Control Solution with Binding Buffer solution. It is recommended to prepare the following dilutions at a total volume of 210 µl (for measuring in duplicate): 1/10, 1/20, 1/40. The suggested method for preparing the dilutions is below:

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Dilution	2X Binding Buffer	Distilled Water	Nuclear Lysate Positive Control Solution	Total Volume
1/10	105 µl	84 µl	21 µl	210 µl
1/20	105 µl	94.5 µl	10.5 µl	210 µl
1/40	105 µl	99.75 µl	5.25 µl	210 µl
Blank	105 µl	105 µl	0 µl	210 µl

• **Wild-Type and Mutant Oligo Controls (optional):** The wild-type mutant oligonucleotide controls are optional and used to determine binding specificity of active transcription factors in samples. If active transcription factors in samples are binding specifically to the wild-type sequence, there will be a reduction in signal in the wild-type control but not in the mutant control. If they are binding non-specifically, there will be reduced signal from both wild-type and mutant controls. It is recommended to use a final concentration of 0.5 nmol of wild-type or mutant oligonucleotide per well. For the oligo control wells, it is recommended to prepare dilutions of nuclear lysate samples at a total volume of 210 µl (for measuring in duplicate) with the addition of approximately 2 µl of wild-type or mutant oligonucleotide. The suggested method for preparing the dilutions is below:

Dilution	2X Binding Buffer	Distilled Water	Sample (Nuclear Lysate)	Wild-Type or Mutant Oligonucleotide	Total Volume
1/20	105 µl	92.4 µl	10.5 µl	2.1 µl	210 µl

2. Aliquot 100 µl of the diluted nuclear lysate positive control into the positive control wells.
3. Aliquot 100 µl of 1X Working Binding Buffer Solution into the blank wells.
4. Aliquot 100 µl of diluted wild-type oligo control into the wild-type oligo control wells.
5. Aliquot 100 µl of diluted mutant oligo control into the mutant oligo control wells.
6. Aliquot 100 µl of diluted sample into the test sample and primary antibody negative control wells.
7. Incubate for 2 hours at room temperature on an orbital shaker.
8. Wash the plate 3 times with 1X Wash Buffer. Fill each well completely with 1X Wash Buffer using a multi-channel Pipette or autowasher (1-2 mins soaking period is recommended). Complete removal of liquid at each step is essential for good performance. After the final wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean absorbent paper towels.
9. Add 100 µl of 1X PPAR-gamma Primary Antibody Working Solution to each well except the primary antibody negative control wells and the blank wells. Add 100 µl of Primary Antibody Diluent Buffer to the primary antibody negative control wells and blank wells. Incubate for 2 hours at room temperature on an orbital shaker.
10. Wash the plate 3 times as directed in Step 8.
11. Add 100 µl of HRP-conjugated Anti-Rabbit IgG antibody to each well and incubate for 1 hour at room temperature on an orbital shaker.
12. Wash the plate 3 times as directed in Step 8.
13. Add 100 µl of TMB Substrate into each well. Cover the plate and incubate at room temperature in dark conditions for 10-30 minutes with gentle shaking on an orbital shaker.
14. Add 100 µl of Stop solution into each well. The colour should change to yellow. Gently tap the plate to ensure thorough mixing. Ensure that there are no fingerprints or water on the bottom of the plate, and that the fluid in the wells is free of bubbles. Measure the absorbance at 450 nm immediately.

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15. If wavelength correction is available, set to 540 nm or 570 nm; otherwise, measure the absorbance at 540 nm or 570 nm and subtract these values from the 450 nm values for analysis. Readings directly at 450 nm without correction may be higher than the actual reading, resulting in less accurate data for concentration determination.

D. Data Analysis

1. For each dilution of the Nuclear Lysate Positive Control, calculate the average OD of the Nuclear Lysate Positive Control wells and blank wells. Subtract the average OD of the blank wells from the average OD of the Nuclear Lysate Positive Control wells to correct for background noise.
2. For each sample (and for each dilution of the sample, if applicable), calculate the average OD of the test sample wells (of the same dilution) and the corresponding primary antibody negative control wells (of the same dilution). Subtract the average OD of the primary antibody negative control wells from the average OD of the corresponding test sample wells to correct for background noise.
3. The OD values from primary antibody negative control wells and blank wells should be lower than 0.2. The OD values for the Nuclear Lysate Positive Control dilutions should generate a gradient for qualitative analysis of the samples. Please note that this assay is not intended for quantitative analysis.

E. Precautions

1. Before using the kit, centrifuge the tubes briefly to bring down any contents trapped in the lid.
2. For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 10 minutes.
3. The wash buffer may crystallise and separate. If this happens, please warm the tube and mix gently to dissolve.
4. Avoid foaming or bubbles when mixing or reconstituting components.
5. It is recommended to assay all controls and samples in duplicate or triplicate.
6. Do NOT let the plate dry out completely during the assay as this will inactivate the biological material on the plate.
7. Ensure plates are properly sealed or covered during incubation steps.
8. Complete removal of all solutions and buffers during wash steps is necessary for accurate measurement readings.
9. To avoid cross contamination do not reuse pipette tips and tubes.
10. Do not use expired components or components from a different kit.
11. The TMB Substrate solution is easily contaminated; work under sterile conditions when handling the TMB substrate solution. The TMB Substrate solution should also be protected from light. Unreacted substrate should be colorless or very light yellow in appearance. Aspirate the dosage needed with sterilized tips and do not dump the residual solution back into the vial.

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