11,12-Dihydroxyeicosatrienoic Acids (11,12-DiHETrE) ELISA Kit

Catalog No.: abx352136

Size: 96T

Range: 1.56 ng/ml - 100 ng/ml

Sensitivity: 0.94 ng/ml

Storage: Store the 96-well plate, Standards, HRP-conjugate reagent and Biotin-conjugated antibody at -20°C, and the rest of the kit components at 4°C for up to 6 months.

Application: For quantitative detection of 11,12-DiHETrE in Serum, Plasma, Tissue Homogenates, Cell Lysates, Cell Culture Supernatants and other biological fluids.

Introduction: 11,12-dihydroxyeicosatrienoic acid (11,12-DiHETrE) is a product of arachidonic acid metabolism. CYP epoxygenase adds an epoxide group (a highly-reactive cyclic ether) to the third carbon-carbon double-bond of arachidonic acid to form 11,12-epoxyeicosatrienoic acid. Epoxygenase hydrolase then breaks the epoxide to form two hydroxyl groups on positions 11 and 12 on the eicosanoid. 11,12-DiHETrE has been shown to inhibit Na+/K+ ATPase in vitro, and may have immunomodulatory functions like many other similar compounds.

Principle of the Assay
This kit is based on a competitive enzyme-linked immuno-sorbent assay technology. 11,12-DiHETrE is pre-coated onto a 96-well plate. The standards, samples and a biotin conjugated antibody specific to 11,12-DiHETrE are added to the wells and incubated. After washing away the unbound conjugates, avidin conjugated to Horseradish Peroxidase is added to each microplate well. After TMB substrate solution is added only wells that contain 11,12-DiHETrE will produce a blue colour product that changes into yellow after adding the stop solution. The intensity of the yellow colour is inversely proportional to the 11,12-DiHETrE amount bound on the plate. The O.D. absorbance is measured spectrophotometrically at 450 nm in a microplate reader, and then the concentration of 11,12-DiHETrE can be calculated.

Kit components
1. One pre-coated 96-well microplate (12 × 8 well strips)
2. Standard: 2 vials
3. Sample/Standard Diluent Buffer: 20 ml
4. Biotin conjugated antibody (100X): 120 µl
5. Antibody diluent buffer: 12 ml
6. HRP-conjugate reagent (100X): 120 µl
7. HRP diluent buffer: 12 ml
8. TMB substrate reagent: 10 ml
9. Stop solution: 10 ml
10. Wash buffer (25X): 30 ml
11. Plate sealer: 5 pieces

Material Required But Not Provided
1. 37°C incubator
2. Microplate reader (wavelength: 450 nm)
3. Multi and single channel pipettes and sterile pipette tips
4. Squirt bottle or automated microplate washer
5. ELISA shaker
6. 1.5 ml tubes to prepare standard/sample dilutions
7. Distilled or deionized water
8. Absorbent filter papers
9. 100 ml and 1 liter graduated cylinders

Protocol

A. Preparation of sample and reagents

1. Sample

Store samples to be assayed within 24 hours at 2-8°C. Alternatively, aliquot and store at -20°C or -80°C for long term. Avoid repeated freeze-thaw cycles.

- **Serum:** Samples should be collected into a serum separator tube. Coagulate the serum by leaving the tube undisturbed in a vertical position overnight at 4°C or at room temperature for up to 60 minutes. Centrifuge at approximately 1000 × g for 20 min. Analyze the serum immediately or aliquot and store at -20°C or -80°C.
- **Plasma:** Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store at -20°C. Avoid hemolysis and high cholesterol samples.
- **Tissue homogenates:** The preparation of tissue homogenates will vary depending upon tissue type – this is just an example. Rinse tissues with ice-cold PBS to remove the excess of blood. Weigh before homogenization. Finely mince tissues and homogenize with a tissue homogenizer on ice in PBS and sonicate the cell suspension. Centrifuge the homogenates at 5000 × g for 5 min and collect the supernatant. Assay immediately or aliquot and store at -20°C.
- **Cell lysates:** Detach adherent cells with trypsin and collect by centrifugation and remove the supernatant. Wash the cells three times in ice-cold PBS and re-suspend cells in PBS. Lyse the cells by ultra-sonification 4 times or freeze at -20°C and thaw to room temperature 3 times. Centrifuge at 1500 × g for 10 min at 2-8°C to remove cellular debris. Collect the supernatant and assay immediately.
- **Cell culture supernatant:** Centrifuge at approximately 2000-3000 × g for 20 min to remove precipitant. Analyze immediately or aliquot and store at -20°C or -80°C.
- **Other biological fluids:** Centrifuge at approximately 1000 × g for 20 min to remove precipitant. Analyze immediately or aliquot and store at -20°C or -80°C.

Note:

» Fresh samples or recently obtained samples are recommended to prevent degradation and denaturalization that may lead to erroneous results. It is recommended to store samples to be used within 5 days at 4°C, within 1 month at -20°C and within 2 months at -80°C.

» Samples should be clear and transparent. Samples must be diluted so that the expected concentration falls within the kit's range.

» Please bring sample slowly to room temperature. Sample hemolysis will influence the result. Hemolyzed specimen should not be used. Samples that contain NaN_3 cannot be detected as it interferes with HRP.

» Always use non-pyrogenic, endotoxin-free tubes for blood collection.

2. Wash buffer

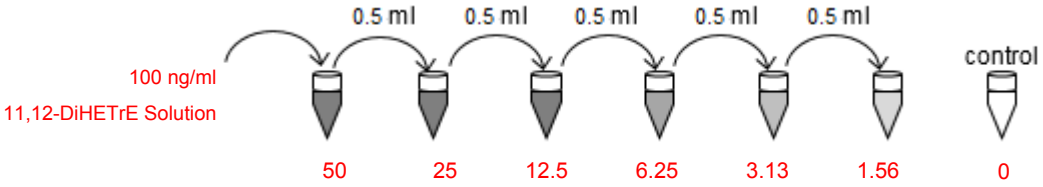
Dilute the concentrated Wash buffer 25-fold (1/25) with distilled water (i.e. add 15 ml of concentrated wash buffer into 360 ml of distilled water).

3. Standard

Preparation of the 11,12-DiHETrE standard: standard solution should be prepared no more than 15 min prior to the experiment. Centrifuge at 10,000×g for 1 minute as the powder may drop off from the cap when opening if you do not spin down. **(Note: Do not dilute the standard directly in the plate).** Once your standard has been reconstituted, it should be used right away. We do not recommend reusing the reconstituted standard.

a.) 100 ng/ml standard solution. Add 1 ml of Sample/Standard diluent buffer into one Standard tube. Allow the reconstituted standard to sit for 15 minutes with gentle agitation prior to carrying out the serial dilutions; avoiding foaming or bubbles.

b.) 50 ng/ml → 1.5625 ng/ml standard solutions: Label 6 tubes with 50 ng/ml, 25 ng/ml, 12.5 ng/ml, 6.25 ng/ml, 3.125 ng/ml and 1.5625 ng/ml. Aliquot 0.5 ml of the Sample / Standard diluent buffer into each tube. Add 0.5 ml of the above 100 ng/ml standard solution into 1st tube and mix thoroughly. Transfer 0.5 ml from 1st tube to 2nd tube and mix thoroughly. Transfer 0.5 ml from 2nd tube to 3rd tube and mix thoroughly, and so on.



Note: Do not vortex the standard during reconstitution, as this will destabilize the protein. Once your standard has been reconstituted, it should be used right away. We do not recommend reusing the reconstituted standard. Please use the diluted Standards for a single assay procedure and discard after use.

4. Preparation of Biotin Detection Reagent working solution: prepare no more than 1 hour before the experiment.
a.) Calculate the total volume of the working solution: 0.05 ml / well × quantity of wells. (Allow 0.1-0.2 ml more than the total volume).
b.) Dilute the Biotin Detection Reagent with Detection Reagent diluent buffer at 1/100 and mix thoroughly. i.e. Add 1 µl of Biotin Detection Reagent into 99 µl of Detection Reagent diluent buffer. Discard after use.

5. Preparation of HRP Conjugated Reagent working solution: prepare no more than 30 min. before the experiment
a.) Calculate the total volume of the working solution: 0.1 ml / well × quantity of wells. (Allow 0.1-0.2 ml more than the total volume).
b.) Dilute the HRP Conjugate Reagent with HRP diluent buffer at 1/100 and mix thoroughly. i.e. Add 1 µl of HRP Conjugate Reagent into 99 µl of HRP diluent buffer. Discard after use.

B. Assay Procedure
Equilibrate the kit components and samples to room temperature before use. It is recommended to plot a standard curve for each test.

1. **Set standard, test sample and control (zero) wells on the pre-coated plate and record their positions. It is recommended to measure each standard and sample in duplicate. Add the solution at the bottom of each well without touching the side walls.**
2. Add 50 µl of the prepared standards solutions into the standard wells. Add 50 µl of Sample / Standard diluent buffer into the control (zero) wells.
3. Add 50 µl of appropriately diluted sample into test sample wells.
4. Immediately add 50 µl of Biotin conjugated antibody working solution into each well. Add the solution at the bottom of each well without touching the side wall.
5. Cover the plate with the plate sealer. Gently tap the plate to mix thoroughly. Incubate at 37°C for 45 minutes.
6. Remove the cover and discard the solution. Wash the plate 3 times with 1X Wash Buffer. Fill each well completely with Wash buffer (350 µl) using a multi-channel pipette or autowasher (1-2 minute 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.
7. Add 100 µl of HRP-conjugate working solution into each well.Cover the plate with a new sealer and incubate at 37°C for 30 minutes.
8. Remove the cover, discard the liquid and wash the plate 5 times with Wash buffer as explained in step 6.
9. Add 90 µl of TMB Substrate into each well. Seal the plate with a cover and incubate at 37°C for 10-20 min. Avoid exposure to light. The incubation time is for reference only, the optimal time should be determined by end user. Do not exceed 30 min.

10. Add 50 µl of Stop solution into each well to stop the enzyme reaction. It is important that the Stop Solution is mixed quickly and uniformly throughout the microplate to inactivate the enzyme completely.
11. 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.

This assay is competitive, therefore there is an inverse correlation between 11,12-DiHETrE concentration in the sample and the absorbance measured. Create a graph with the log of the standard concentration (y-axis) and absorbance measured (x-axis). Apply a best fit trendline through the standard points. Use this graph calculate sample concentrations based on their OD values. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Note: If the samples measured were diluted, multiply the dilution factor by the concentrations from interpolation to obtain the concentration before dilution.

C. Precautions

1. Before using the kit, centrifuge the tubes briefly to bring down the contents trapped in the lid.
2. If crystals have formed in the concentrated Wash Buffer, warm to room temperature and mix gently until the crystals have completely dissolved.
3. Avoid foaming or bubbles when mixing or reconstituting components. Prepare the Standard dilutions within 15 min of use and discard any unused working standards. For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 10 minutes.
4. Do not let the wells uncovered for extended periods between incubation. Once reagents are added to the wells, avoid letting the strips dry as this can inactivate the biological material on the plate. Incubation time and temperature must be controlled.
5. Ensure plates are properly sealed or covered during incubation steps.
6. Complete removal of all solutions and buffers during wash steps is necessary for accurate measurement readings.
7. Do not reuse pipette tips and tubes to avoid cross contamination.
8. 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.

D. Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, medium and high levels of 11,12-DiHETrE were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, medium and high levels of 11,12-DiHETrE were tested on 3 different plates, 8 replicates in each plate.

CV (%) = (Standard Deviation / mean) × 100

Intra-Assay CV: ≤ 10%

Inter-Assay CV: ≤ 10%

Sample Recovery (after spiking)

Sample Type	Range	Average Recovery
Serum (n=5)	89-104%	97%
EDTA Plasma (n=5)	91-101%	96%
Cell Culture Media (n=5)	92-109%	99%

Linearity

Sample Type	1:2	1:4	1:8	1:16
Serum (n=5)	87-101%	88-102%	90-103%	87-98%
EDTA Plasma (n=5)	99-115%	90-103%	87-100%	94-109%
Cell Culture Media (n=5)	97-112%	98-115%	101-114%	94-110%

E. Typical Data & Standard Curve

Typical Standard Curve Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Concentration ng/ml	0	1.5625	3.125	6.25	12.5	25	50	100
OD450	2.206	1.874	1.614	1.267	0.905	0.614	0.426	0.32