11,12-DHET ELISA kit

Catalog Number: **DH4/DH14/DH24/DH104**
Store at -20°C.
FOR RESEARCH USE ONLY

V. 10232012

Introduction

This competitive ELISA kit is for determination of 11,12-DHET levels in biological samples. The level of 11,12-DHET or 11,12-DHET epitope has been shown to exhibit correlation with hypertension in rodents (1,2,3). 11,12-DHET is a representative metabolite of cytosolic epoxide hydrolase-mediated metabolism of EETs, which are generated by arachidonic acid epoxygenase activity of cytochrome P_{450}'s (4).

This kit can be used for the determination of 11,12-DHET in serum, plasma, cells, and tissues following proper isolation and purification. Instructions are provided as to the proper isolation and purification in the following pages.

This competitive ELISA kit, based on competition between 11,12-DHET epitope and 11,12-DHET-HRP conjugate for a limited number of binding sites available from the anti-11,12-DHET antibody, which is coated to the wells of the 96 well ELISA plate. The conjugate concentration is held as a constant in each well, while the concentration of the 11,12-DHET is variable, based on the concentration of the sample or standard. Thus the amount of the 11,12-DHET conjugate which is able to bind to each of the wells is inversely proportional to the concentration of 11,12-DHETin the standard or sample. The amount of the conjugate which is bound to each well is then determined by the amount of color obtained, when TMB is added. The TMB reacts with the HRP available in the well. With the addition of sulfuric acid, the blue colored product is converted into a yellow colored product, which can be read on a plate reader at 450 nm.

Storage and Stability

This kit will obtain optimal results if all of the components are stored at the proper temperature prior to use. Items should be stored at the designated temperatures upon receipt of this kit. All components are stored below -20°C and should not be re-frozen and thawed more than necessary.

Materials Provided

Part Number	Item	Description	Quantity
1	11,12-DHET ELISA Plate	Solid 96-well plate coated with anti-11,12-DHET antibody in each well	1
2	11,12-DHET Standard (2 µL)	Stock standard at a concentration of 1 mg/mL	1
3	11,12-DHET-HRP Conjugates (12 µL)	1000 X concentrated solution	1
4	Sample Dilution Buffer (25 mL)	10 X solution of Tris-buffered saline with preservatives	1
5	HRP Buffer (15 mL)	1 X solution of Tris-buffered saline with preservatives	1
6	Wash Buffer Solution (25 mL)	10 X solution of Tris-buffered saline with detergents and preservatives	1
7	TMB Substrate (24 mL)	A solution of TMB (tetra methyl benzadine)	1

Additional Required Materials (Not Provided)

- Plate reader with a 450 nm filter
- An 8-channel adjustable pipetter and an adjustable pipetter
- Storage bottles
- Costar® cluster tubes (1.2 mL) and microcentrifuge tubes
- Deionized water

Precautions

- Please read all instructions carefully before beginning the assay.
- The reagents in this kit have been tested and formulated to perform optimally. This kit may not perform correctly if any of the reagents are replaced or any of the procedures are modified.
- This kit is intended for research use only and is not to be used as a diagnostic.

Procedural Notes

- Remove all of the reagents required, including the TMB, and allow them to equilibrate to room temperature before proceeding with the assay.
- It is necessary to thoroughly mix the concentrated buffer solutions. A stir bar is contained within each buffer solution.

Sample Preparations

There are different protocols for isolating and purifying 11,12-DHET depending on the medium in which it is in. Listed below are the different protocols. For optimal results follow the appropriate protocol based on the biological sample present.

11,12-DHET measurement in cells

1. Collect and homogenize and/or sonicate the cells using a solution containing a final concentration of ~0.1 mM TPP (triphenyphosphine, 0.03-0.05 mg/mL). TPP is an antioxidant, which looks like a precipitate in samples because it does not easily dissolve. Before using the stored samples containing TPP, spin samples to separate the precipitated TPP from sample solution.
2. Acidify the whole homogenized cells with acetic acid to a pH of approximately 3-4. Measure using standard pH paper.
3. Extraction with ethyl acetate. Add an equal volume of ethyl acetate to the homogenized cells and vortex very well. Place the upper organic phase into a fresh clean tube after centrifugation. Then add another equal volume of ethyl acetate to the homogenized cells to start the second-time extraction. It is strongly recommended that extraction is performed three times.
4. Evaporate the pooled ethyl acetate from the extractions until all has dried up under argon or nitrogen gas.
5. **Saponification if needed (see below)**
6. Add 10 µL to 20 µL ethanol, or N, N-dimethyl-formamide (DMF), to dissolve the dried-up residue from above step #4. Add 0.5 mL of 1x Sample Dilution Buffer (provided in kit). Load 100 µL in each well, in triplicates, on the ELISA plate. (Note: We recommend measuring a different dilution of sample in attempt to fit the results to the standard curve. e.g., add 3 wells by 50 µL of the rest of sample plus 50 µL of 1x Sample Dilution Buffer, and 3 wells of 10 µL of the rest of sample and 90 µL of 1x Sample Dilution Buffer.)
7. Perform the ELISA for 11, 12-DHET (according to the instructions of the manufacturer).

Saponification (to cleave fatty acid from glycerol backbone):

1. Dissolve dried fatty acids (obtained from 3X ethyl acetate extractions) in 2mL of 20% KOH solution (make working solution: 1 mL of 2 M KOH + 4 mL methanol with final concentration of KOH = 0.4 N).
2. Vortex and incubate for 1 h at 50°C.
3. Add 1.5X H_2O to the solution and adjust pH with 20% formic acid to pH ~5.
4. Re-extract the solution with ethyl acetate (1 part aqueous solution + 1 part ethyl acetate) and dry.

11,12-DHET measurement in tissues

1. Homogenize 1 g of tissue, 4 mL of H_2O, and 0.01 mg TPP.
2. Acidify the homogenate by adding 8 µL of acetic acid to each homogenate.
3. Extract with an equal amount of ethyl acetate, vortex thoroughly, spin down, and collect the organic phase. Repeat this extraction twice more and combine all of the organic phases.
4. Dry the organic substance with argon or nitrogen gas.
5. **Saponification if needed (see 11,12-DHET measurement in cells)**
6. Dissolve the dried residue from above step #4 with ethanol or DMF. (Add approximately 20 µL of ethanol or DMF to reconstitute the dried-up residue).
7. Dilute further with 1x Sample Dilution Buffer. Add approximately 0.5 mL of 1x Sample Dilution Buffer and centrifuge at 10,000 rpm for five minutes at room temperature. The supernatant will be used for ELISA.
8. Perform the ELISA for 11, 12-DHET (according to the instructions of the manufacturer).

11,12-DHET measurement in <u>plasma or serum</u>

Below are two protocols for measuring 11,12-DHET in plasma or serum using starting volumes of 1.0 or 1.8 mL. Starting volumes below 1.0 mL can also be used (i.e. for mouse studies) for measuring 11,12-DHET by making appropriate adjustments to volumes and dilutions. Please contact customer service at 313-961-1606 for assistance.

I. Protocol for 1.0 mL <u>plasma or serum</u>

1. Combine 1.0 mL of plasma (adjusted with approximately 12 µL of acetic acid to pH 4) and 1.0 mL of ethyl acetate. Vortex thoroughly. Centrifuge at 2000 rpm for ten minutes at 22°C. Three phases should result:
    i. Upper organic phase – ethyl acetate phase (lipoproteins)
    ii. Interphase – proteins
    iii. Lower phase – aqueous phase
2. Collect the upper organic phase (a) and set aside.
3. Discard the interphase. Transfer the lower phase with a glass pipette to a new tube, and repeat the ethyl acetate extraction step 2 more times.
4. Evaporation of pooled organic phase: There should be approximately 3 mL of the ethyl acetate phase (a). Dry the pooled organic phase in a Speedvac to get the extracted sediment (b).
5. Saponification (to cleave fatty acid from glycerol backbone): Dissolve the dried residues (b) in 2 mL of 20% KOH solution (for preparation see 11,12-DHET measurement in cells). Vortex thoroughly and incubate for 1 h at 50°C. This will yield an aqueous solution (c).
6. Dilute 2 mL of the aqueous solution (c) with 3 mL of H_2O. Adjust the pH using 20% formic acid (132 µL) to pH~5.5. Add ethyl acetate (1 part aqueous solution (c) + 1 part ethyl acetate), vortex thoroughly, and centrifuge at 2000 rpm for ten minutes at 22°C. Repeat the procedure twice more using an equal volume of ethyl acetate per sample. Collect the upper phase containing saponified lipids.
7. Dry the pooled ethyl acetate upper phase (d) and dry in a Speedvac, yielding the dried sample-sediment (e). Store the sediment (e) at -20°C. For ELISA assay, dissolve the sediment (e) in 20 µL of ethanol, then add 380 µL of 1X Sample Dilution Buffer, pH 7.4. *(Please note that the 10X Sample Dilution Buffer that is supplied with the ELISA kit must be diluted 10-fold).*

8. When calculating the concentration, consider the dilution factor. In this case, 400 µL total sample volume from 1.0 mL plasma (2.5-fold concentration) you must divide your calculated result by 2.5.
9. Perform the ELISA for 11,12-DHET (according to the instructions of the manufacturer).

II. **Protocol for 1.8 mL <u>plasma or serum</u>** ***(Following this procedure, the user will have approximately 70 uL of material left-over from step 8. This material can be stored at -20^{o}C and be used for a second measurement following a 5X dilution).***

1.Combine 1.8 mL of plasma (adjusted with approximately 20 µL of acetic acid to pH 4) and 1.8 mL of ethyl acetate. Vortex thoroughly. Centrifuge at 2000 rpm for ten minutes at 22°C. Three phases should result:

i. Upper organic phase – ethyl acetate phase (lipoproteins)
ii. Interphase – proteins
iii. Lower phase – aqueous phase

2. Collect the upper organic phase (a) and set aside.
3. Discard the interphase. Transfer the lower phase with a glass pipette to a new tube, and repeat the ethyl acetate extraction step 2 more times.
4. Evaporation of pooled organic phase: There should be approximately 5-6 mL of the ethyl acetate phase (a). Dry the pooled organic phase in a Speedvac to get the extracted sediment (b).
5. Saponification (to cleave fatty acid from glycerol backbone): Dissolve the dried residues (b) in 2 mL of 20% KOH solution (for preparation see 11,12-DHET measurement in cells). Vortex thoroughly and incubate for 1 h at 50°C. This will yield an aqueous solution (c).
6. Dilute 2 mL of the aqueous solution (c) with 3 mL of H_2O. Adjust the pH using 20% formic acid (132 µL) to pH~5.5. Add ethyl acetate (1 part aqueous solution (c) + 1 part ethyl acetate), vortex thoroughly, and centrifuge at 2000 rpm for ten minutes at 22°C. Repeat the procedure twice more using an equal volume of ethyl acetate per sample. Collect the upper phase containing saponified lipids.
7. Dry the pooled ethyl acetate upper phase (d) and dry in a Speedvac, yielding the dried sample-sediment (e). Store the sediment (e) at -20°C. For ELISA assay, dissolve the sediment (e) in 20 µL of ethanol, then add 130 µL of 1X Sample Dilution Buffer.
8. For the competitive 11,12-DHET ELISA, the above 150 µL sample needs to be further diluted: Dilute 1:4 (e.g., 80 µL sample + 320 µL 1x Sample Dilution Buffer). Check the final pH (should be pH 7.4). When calculating the concentration, consider the dilution factor. In this case, 150 µL total sample volume from 1.8 mL plasma (12-fold concentration) and then, 80 uL sample in 400 µL SDB (5-fold dilution). Since, the samples are concentrated 2.4-fold; to get the actual concentration, you must divide by 2.4.
9. Perform the ELISA for 11,12-DHET (according to the instructions of the manufacturer).

11,12-DHET measurement in <u>urine.</u>

1. Extraction using ethyl acetate is not necessary. It is recommended that the urine sample be diluted 4-fold with 1X sample dilution buffer and 100 uL of sample added directly to the ELISA plate well.
2. However, extraction of urine with ethyl acetate can be performed if desired. See protocols for extraction with ethyl acetate above.
3. When calculating the concentration, consider the dilution factor

<u>Please contact us for urine or plasma extraction procedure or human study.</u>

Assay Preparations

The solid 96-well plate and TMB solution are provided ready to use. The preparations of other assay reagents are detailed below.

Wash Buffer: Mix the solution with a stir bar, applying low heat until a clear colorless solution is obtained. Dilute the entire contents of the Wash Buffer Concentrate (25 mL) with 225 mL of deionized water to yield a final volume of 250 mL of 1 X Wash Buffer. This can then be refrigerated for the entire life of the kit.

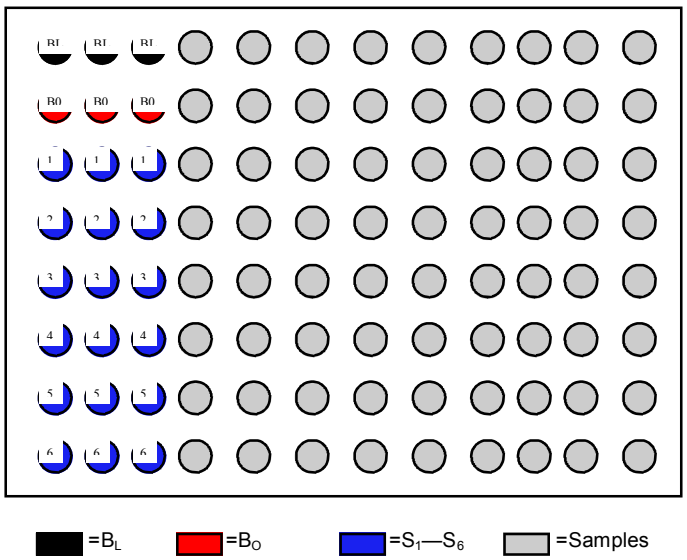
HRP Conjugate: Dilute 1 vial of the 11,12-DHET-HRP conjugate (0.012 mL) with 12.00 mL of 1 X HRP buffer. One vial makes enough conjugate for one plate. The conjugate must be used the same day and should not be stored for later use.

Standards: Label 5 microtubes as Standard 1 through Standard 5. Dilute the entire contents of Sample Dilution Stock buffer (25 mL) with 225 mL deionized water to yield a final volume of 250 mL of 1 X Sample Dilution Buffer. Add 0.9 mL of the Sample Dilution Buffer to the microtubes for Standards 1 to 5. Spin down the enclosed 11,12-DHET standard vial (2 µL, filled with inert gas) and add 1.998 mL of Sample Dilution Buffer to obtain 2 mL of solution. Label this Standard 6. Add 0.1 mL of the Standard 6 to the microtube labeled Standard 5 and mix thoroughly. Next, add 0.1 mL of Standard 5 into the microtube labeled Standard 4 and mix thoroughly. Continue to serially dilute the standards using 1:10 dilutions for the remaining standards.

Samples: Samples can be directly diluted into the 1 X Sample Dilution Buffer if it is in solution. For extracted and dried samples, it is recommended to dissolve the dried-up samples with a minimal amount of ethanol of N, N-dimethyl-formanmide (DMF, 10 µL to 20 µL) and vortex well. Before ELISA assay, add 100 µL of 1 X Sample Dilution Buffer to make the stock sample solution ready for quantification with ELISA. The stock sample solution can be further diluted to a proper range of concentration for ELISA test.

Performing the Assay

Plate Setup: Each plate must contain a minimum of three blank wells (B_L), three maximum binding wells (B_O), and a six point standard curve (S_1-S_6). Each sample should be assayed in triplicate. A suggested plate format is shown below:



Standard Dilutions Table

Standards	Final Concentration (pg/mL)	Add Sample Dilution Buffer (mL)	Serial Dilutions Procedure
No. 6	1,000,000	1.998	2 µL of stock solution.
No. 5	100,000	0.9	Add 0.1 mL of No. 6
No. 4	10,000	0.9	Add 0.1 mL of No. 5
No. 3	1,000	0.9	Add 0.1 mL of No. 4
No. 2	100	0.9	Add 0.1 mL of No. 3
No. 1	10	0.9	Add 0.1 mL of No. 2

Assay Procedure

Step 1: Load 200 microliters of Sample Dilution Buffer into the blank (B_L) wells and 100 microliters of Sample Dilution Buffer into the maximum binding (B_O) wells.

Step 2: Load 100 microliters of each of the standards into the appropriate wells.

Step 3: Load 100 microliters of each of the samples into the appropriate wells.

Step 4: Load 100 microliters of the diluted 11,12-DHET-HRP conjugate in the B_O wells, the standard wells, and the sample wells. Do NOT add HRP conjugate into the B_L wells.

Step 5: Incubate the plate at room temperature for two hours.

Step 6: Wash the plate three times with 400 microliters of the diluted Wash Buffer per well.

Step 7: After the last of the three wash cycles pat the plate dry onto some paper toweling.

Step 8: Add 200 microliters of the TMB substrate to all of the wells (including B_L wells).

Step 9: Incubate the plate at room temperature for 15-30 minutes.

Step 10: Add 50 micoliters of 2 N sulfuric acid to all of the wells.

Step 11: Read the plate at 450 nm.

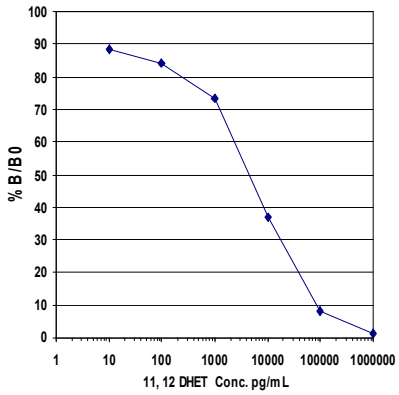
Calculating the Results

Most plate readers provide data reduction software that can be used to plot the standard curve and determine the sample concentrations. If your plate reader does not have this option, then a data reduction program can be used (4 parameter of log-log curve fit).

If you do not have these options, the results can be obtained manually as follows:

1. Average the absorbance readings from the blanks and subtract that value from each well of the plate to obtain the corrected readings. (Note: Some plate readers do this automatically. Consult the user manual of your plate reader.)
2. Average the corrected absorbance readings from the B_O wells. This is your maximum binding.
3. Calculate the $\%B/B_O$ for Standard 1 by averaging the corrected absorbance of the two S_1 wells, divide the average by the maximum binding, then multiply by 100. Repeat this formula for the remaining standards.
4. Plot the $\%B/B_O$ versus the concentration of 11,12-DHET from the standards using semi-log paper.
5. Calculate the $\%B/B_O$ for the samples and determine the concentrations, utilizing the standard curve.
6. Multiply the concentrations obtained for each of the samples by their corresponding dilution factor.

Typical Results



The data shown here is an example of typical results obtained using the Detroit R & D 11,12-DHET ELISA kit. These results are only a guideline, and should not be used to determine values from your samples. The user must run their own standard curve every time.

B_L wells = 0.066
B_O wells = 1.337

Standard	Concentration	O.D.	%B/B_O
No. 1	10 pg/mL	1.179	88.2
No. 2	100 pg/mL	1.126	84.2
No. 3	1,000 pg/mL	0.981	73.4
No. 4	10,000 pg/mL	0.495	37.1
No. 5	100,000 pg/mL	0.108	8.1
No. 6	1,000,000 pg/mL	0.019	1.4

Troubleshooting

No color present in standard wells.

- The HRP conjugate was not added. Redo the assay and add the conjugate at the proper step.
- The HRP conjugate was not incubated for the proper time. Redo the assay and incubate for the proper time.

No color in any wells, including the TA wells.

- The TMB substrate was not added. Add substrate.
- The TMB substrate was not incubated for the proper time. Continue incubation until desired color is reached.

The color is faint.

- One or all of the incubation times were cut short. Redo the assay with the proper incubation times.

- The TMB substrate was not warmed up to room temperature. Redo the assay making sure all reagents are at room temperature.
- The lab is too cold. Be sure the lab temperature is between 21-27°C and redo the assay.

The background color is very high.

- The TMB substrate has been contaminated. Redo the assay with a fresh bottle of substrate.

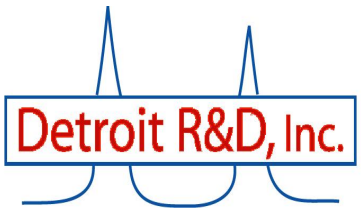
Scattered O.D. obtained from the sample.

- Redo assay using an 8-channel pipetman making sure that 8 channels are equal volume while loading.

References

1. Kim et al. Two divisional US Patents: 6,440,682 and 6,534,282 issued on 8/27/2002 and 3/18/2003, respectively.
2. Garson et al. Computational characterization of a series of eicosanoids. Lett. Drug Design and Discovery 2, 322, 2005.
3. Sinal et al. Targeted disruption of soluble epoxide hydrolase reveals a role in blood pressure regulation. J. Biol. Chem. 275, 40504, 2000.
4. Yu et al. Soluble epoxide hydrolase regulates hydrolysis of vascoactive epoxyeicosatrienoic acids. Circulation Research 87, 992, 2000.
5. Makita et al. Cytochrome P450, the arachidonic acid cascade, and hypertension: new vistas for an old enzyme system. FASEB J. 10, 1456, 1996, and references therein.
6. Spiecker et al. Risk of coronary artery disease associated with polymorphism of the cytochrome P450 epoxygenase CYP2J2. Circulation 110, 2132, 2004.
7. Wang et al. Cytochrome P450 2J2 promotes the neoplastic phenotype of carcinoma cells and is up-regulated in human tumors. Cancer Res. 65, 4707, 2005.
8. Wang et al. Cytochrome P-450 Epoxygenase Promotes Human Cancer Metastasis. Cancer Res. 67, 6665-6667, July 15, 2007.
9. Zeldin et al. Cytochrome P-450 epoxygenases protect endothelial cells from apoptosis induced by tumor necrosis factor-a via MAPK and PI3K/Akt signaling pathways. Am J. Physiol Heart Circ Physiol 293: H142-H151, 2007.
10. Alkayed et al. Polymorphisms in the Human Soluble Epoxide Hydrolase Gene EPHX2 Linked to Neuronal Survival after Ischemic Injury. J. Neurosci. 27(17): 464-4649, April 25, 2007.
11. Chen et al. Synergistic effect of cytochrome P450 epoxygenase CYP2J2*7 polymorphism with smoking on the onset of premature myocardial infraction. Atherosclerosis 195, 199-206, 2007.
12. D. Wang, T. Hirase, T. Nitto, M. Soma, K. Node. Eicosapentaenoic acid increases cytochrome P450 2J2 gene expression and epoxyeicosatrienoic acid production via peroxisome proliferator-activated receptor γ in endothelial cells. J. Cardiol. 54, 368-374, 2009.

Warranty



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