11,12-EET/DHET ELISA kit

Catalog Number: **DH5/DH15/DH25**
Store at -20°C.
FOR RESEARCH USE ONLY
V.05262010

Introduction

It is well known that arachidonic acid (AA) will be converted to EET by P_{450} arachidonic acid epoxygenase (AA epoxygenase) and EET will be converted to DHET by soluble epoxide hydrolase (sEH) *in vivo*. Cytochrome P_{450} 2J2 (CYP2J2) is a predominant human AA epoxygenase that produces all four EETs. Our EET/DHET kit can be used to measure EET levels in cultured cells which express sEH (1).

DH5, DH15, or DH25 to be used for 11,12-EET/DHET measurement is the same kit as DH4, which is used for the measurement of 11,12-DHET. The only difference with DH5, DH15, and DH25 compared with DH4 is the sample preparation step. Instructions are provided as to the proper isolation and purification in the following pages.

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

EET+DHET can be measured after chemically changing EET to DHET. However, if the EET in cells or in blood is changed to DHET by abundantly expressed soluble epoxide hydrolase, measurement of DHET without chemically changing EET to DHET is suitable.

However, when P450 2C23 activity of the rat microsomes was measured, the rat microsomes were incubated with arachidonic acid (substrate of P450 2C23) and, then, EET + DHET levels in the reaction mixture were measured after acid hydrolysis of EET to DHET, which was indicative of P450 2C23 activity.

There are three different protocols which can be used to convert EET into DHET for measurement using the competitive ELISA kit. For optimal results please choose the protocol which fits your sample best.

Protocol #1: EET formation activity measurement

1. Collect and homogenize and/or sonicate the cells using a solution containing a final concentration of 0.1 mM TPP (triphenylphosphine). TPP is an antioxidant, which looks like precipitate in samples because it does not easily dissolve. Before using the stored samples with TPP, spin to separate the TPP from the samples.
2. Acidify the whole homogenized cells with acetic acid to a pH of approximately 3-4. Measure using standard pH paper. (Be careful when changing pH by adding 1 µL of acetic acid at a time.)
3. Extract with ethyl acetate. Add an equal volume of ethyl acetate to the homogenized cells, and vortex thoroughly. Transfer the upper organic phase into a fresh clean tube after centrifugation. Then add another equal volume of ethyl acetate to the homogenized cells and repeat the extraction two more times.
4. Evaporate the pooled ethyl acetate until all is dried up under argon gas.
5. Add 20 µL of ethanol or N, N-dimethyl-formamide (DMF) to dissolve the dried up residue for reconstitution. Add 0.5 mL 1x Sample Dilution Buffer (provided in the kit) to make a solution. Load 100 µL into each well in triplicate on the ELISA plate. (Note: We recommend measuring a different dilution of the sample in an attempt to fit the results to the standard curve. e.g. Add 50 µL of the rest of the sample plus 50 µL 1x Sample Dilution Buffer to three wells plus add 10 µL of the rest of the sample plus 90 µL of 1x Sample Diltuion Buffer to three wells.)
6. Perform the ELISA for 11,12-DHET (according to the instructions of the manufacturer).

Protocol #2: Free EET + DHET formation activity measurement

1. Biological samples have to be collected in TPP (triphenylphosphine) with a final concentration of 0.1 mM. TPP is an antioxidant, which looks like precipitate in samples because it does not easily dissolve. Before using the stored samples with TPP, spin to separate the TPP from the samples.
2. Acidify the samples with acetic acid to a pH of approximately 3-4. After acidification, the samples are extracted three times with ethyl acetate. For each extraction, add an equal volume of ethyl acetate to the sample, vortex thoroughly, spin down, and collect the organic phase. After extracting three times from the same sample, pool the collected organic phases (ethyl acetate) and evaporate under argon gas.
3. Dissolve the above dried up residue in 20 μL of ethanol then add 20 μL of acetic acid to make the pH approximately 3-4. In the acidic conditions EET is hydrolyzed to DHET. The reaction usually takes 12 h at 45°C or 18 h (overnight) at room temperature. The reaction vial has to be flushed with argon and kept under an argon blanket. (An argon blanket is like a pouch to keep an argon gas flow during the hydrolysis.) If an argon blanket is not available at your place, you can add clean powdered dry ice to get rid of residual oxygen.
4. After the reaction, add 1.5X water to the sample and extract the sample three times with equal volume of ethyl acetate (vortex well, spin down and collect the organic phase). After three times of extraction, pool all the organic phase (ethyl acetate) together and evaporate under argon or nitrogen.
5. For ELISA assay, dissolve the sediment in 20 uL of ethanol or DMF (vortex thourghly), then add 130 uL of 1x Sample Dilution Buffer to make stock solution. The stock sample solution can be diluted in a proper range of concentration for ELISA test. Check the final pH (should be pH 7.4).
6. Use the 11,12-DHET ELISA kit to measure DHET, which includes DHET converted from EET. At the same time, measure the DHET level without hydrolysis of EET in the same sample. Subtract that value from the EET + DHET level and you will obtain the EET level in the sample.

Protocol #3: Free and esterified EET + DHET formation activity measurement

1. Biological samples have to be collected in TPP (triphenylphosphine) with a final concentration of 0.1 mM. TPP is an antioxidant, which looks like precipitate in samples because it does not easily dissolve. Before using the stored samples with TPP, spin to separate the TPP from the samples.
2. Acidify the samples with acetic acid to a pH of approximately 3-4. After acidification, the samples are extracted three times with ethyl acetate. For each extraction, add an equal volume of ethyl acetate to the sample, vortex thoroughly, spin down, and collect the organic phase. After extracting three times from the same sample, pool the collected organic phases (ethyl acetate) together and evaporate under argon gas.
3. To cleave the esterified eicosanoids, 2 mL of 20% KOH was added and mixed very well. The mixture was incubated at 50°C for one hour. [Prepare a 20% KOH solution from 1 mL 2M KOH and 4 mL methanol (final concentration KOH = 0.4 N)].
4. Dilute 2 mL of the aqueous solution with 3 mL of H_2O. Adjust the pH using 20% formic acid to pH~5. Add ethyl acetate (1 part aqueous solution + 1 part ethyl acetate), vortex thoroughly, and centrifuge at 2000 rpm for ten minutes at 22ºC. Repeat the procedure twice more using an equal volume of ethyl acetate per sample. Pool all the organic phase (ethyl acetate) together and evaporate under argon gas.
5. Dissolve the dried residue in a minimal amount of ethanol (~20 uL), add 20 μL of acetic acid to make a pH of approximately 3-4. In the acidic conditions EET is hydrolyzed to DHET. The reaction usually takes 12 h at 45°C or 18 h (overnight) at room temperature. The reaction vial has to be flushed with argon and kept under an argon blanket. (An argon blanket is like a pouch to keep an argon gas flow during the hydrolysis.) If an argon blanket is not available at your place, you can add clean powdered dry ice to get rid of residual oxygen.
6. After reaction, add 1.5x water to the sample and extract the sample three times with equal volume of ethyl acetate. For each extraction, vortex thoroughly and spin down and collect the organic phase. After three times of extraction, pool all the organic phase (ethyl acetate) together and evaporate under argon.
7. For ELISA, dissolve the sediment in 20 uL of ethanol or DMF (vortex thourghly), then add 130 uL of 1x Sample Dilution Buffer to make stock solution. The stock sample solution can be diluted in a proper range of concentration for ELISA test. Check the final pH (should be pH 7.4).
8. Use the 11,12-DHET ELISA kit to measure DHET, which includes DHET converted from EET. At the same time, measure the DHET level without hydrolysis of EET in the same sample, and subtract it from the EET + DHET level. Then you will obtain the EET level in the sample.

References

1. Cancer Res. 2005; 65:4707-15.
2. *Circulation*. 2004; 110:2132
3. *Letters in Drug Design & Discovery*. 2005; 2:239, etc.

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.

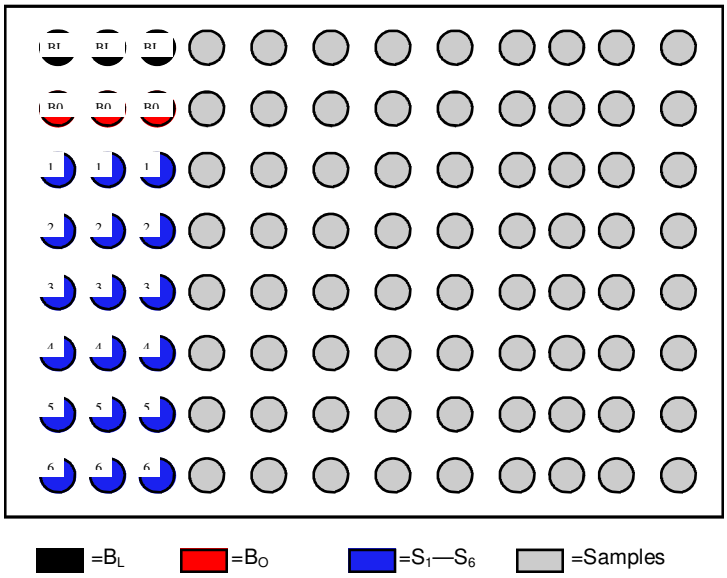
HRP Conjugate: Dilute 1 vial of the 11,12-DHET-HRP conjugate (0.012 mL) with 12.00 mL of 1 X HRP buffer. One vial makes enough conjugate for one plate. The conjugate must be used the same day and should not be stored for later use.

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.

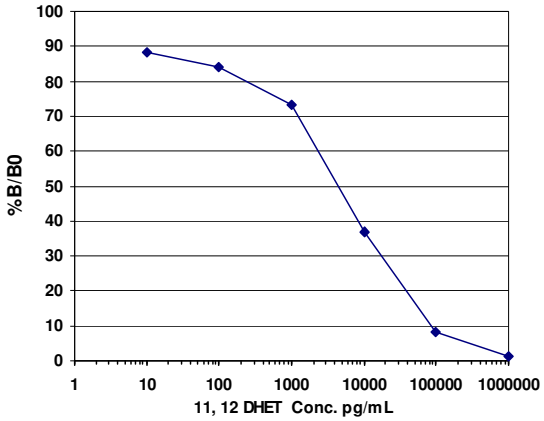
Calculating the Results

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.

Warranty

Detroit R&D, Inc., makes no warranty of any kind expressed, or implied, including, but not limited to the warranties of fitness for a particular purpose and merchantability.

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