



EAGLE
BIOSCIENCES

14,15-EET/DHET ELISA Kit

Catalog Number:

14E39-K01 (1 x 96 wells)

14E39-K02 (2 x 96 wells)

14E39-K05 (5 x 96 wells)

14E39-K010 (10 x 96 wells)

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

v. 3.0 (effective 11May23)

Eagle Biosciences, Inc.

20A Northwest Blvd., Suite 112, Nashua, NH 03063

Phone: 617-419-2019 Fax: 617-419-1110

www.EagleBio.com



INTENDED USE

The Eagle Biosciences **14,15 EET / DHET ELISA Assay** kit is intended for the quantitative determination of **14,15 EET / DHET** in biological samples by enzyme linked immunoassay (ELISA). The Eagle Biosciences **14,15 EET / DHET ELISA Assay kit** is for research use only and not to be used in diagnostic procedures

ASSAY BACKGROUND

It is well known that arachidonic acid (AA) will be converted to EET by P₄₅₀ arachidonic acid epoxygenase (AA epoxygenase) and EET will be converted to DHET by soluble epoxide hydrolase (sEH) in vivo. Cytochrome P₄₅₀ 2J2 (CYP2J2) is a predominant human AA epoxygenase that produces all four EETs. In human carcinoma cells, rAAV-mediated over expression of CYP2J2 resulted in a marked increase in 14,15-DHET level in cell plasma, whereas rAAV-anti2J2-mediated silence of CYP2J2 expression significantly decreased its production (1). The Eagle Biosciences 14, 15 EET/DHET ELISA Assay kit can be used to measure EET levels in cultured cells which express sEH (1).

DH2, DH12, or DH22 to be used for 14,15-EET/DHET measurement is the same kit as DH1, which is used for the measurement of 14,15-DHET. The only different with DH2, DH12, and DH22 compared with DH1 is the sample preparation step. Instructions are provide as to the proper isolation and purification in the following pages.

MATERIALS PROVIDED

Item	Quantity	Description
14,15-DHET ELISA Plate	1	Solid 96-well plate coated with anti14,15DHET antibody in each well
14,15-DHET Standard (2 µL)	1	Stock standard at a concentration of 1 mg/mL
14,15-DHET-HRP Conjugates (12 µL)	1	1000 X concentrated solution
Sample Dilution Buffer (25 mL)	1	10 X solution of Tris-buffered saline with preservatives
HRP Buffer (15 mL)	1	1 X solution of Tris-buffered saline with preservatives
Wash Buffer Solution (25 mL)	1	10 X solution of Tris-buffered saline with detergents and preservatives
TMB Substrate (24 mL)	1	A solution of TMB (tetra methyl benzadine)

MATERIALS REQUIRED BUT 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



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 stored below -20°C and should not be re-frozen and thawed more than necessary

PRECAUTIONS

- Please read all instructions carefully before beginning the 11,12-EET/DHET ELISA Assay kit.
- The reagents in the 14,15 EET / DHET ELISA Assay kit have been tested and formulated to perform optimally. The 14,15 EET / DHET ELISA Assay kit may not perform correctly if any of the reagents are replaced or any of the procedures are modified.
- The 14,15 EET / DHET ELISA Assay 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.

For example, when 14,15-DHET levels were measured in urine samples obtained from Spontaneously Hypertensive rats, 14,15-DHET levels in the urine were measured without changing EET to DHET. High 14,15-DHET levels were indicative of increased soluble epoxide hydrolase activity of the rat (thus soluble epoxide hydrolase-dependent hypertension).

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 14,15 EET / DHET ELISA Assay 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 μ L 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 μ L 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 14,15EET/DHET ELISA Assay kit) to make a solution. Load 100 μ L 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 μ L of the rest of the sample plus 50 μ L 1x Sample Dilution Buffer to three wells plus add 10 μ L of the rest of the sample plus 90 μ L of 1x Sample Dilution Buffer to three wells.)
6. Perform the ELISA for 14,15-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 μ L of ethanol then add 20 μ L 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 μ L of ethanol or DMF (vortex thoroughly), then add 130 μ L of 1x Sample Dilution Buffer to make stock solution. The stock sample solution can be diluted in a proper range of concentration for the 14,15-EET/DHET ELISA test. Check the final pH (should be pH 7.4).
6. Use the 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 H₂O. 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 22°C. 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 thoroughly), 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 the assay test. Check the final pH (should be pH 7.4).
8. Use the 14,15-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.

ASSAY PREPARATION

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 14,15-EET / DHET ELISA Assay kit.

HRP Conjugate: Dilute 1 vial of the 14,15-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 14,15-EET/DHET ELISA Assay Kit

5/10

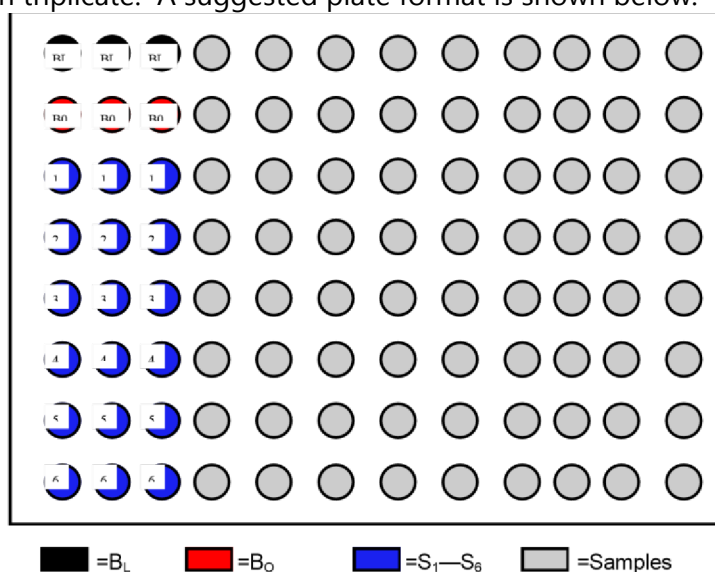


mL of 1 X Sample Dilution Buffer. Add 0.9 mL of the Sample Dilution Buffer to the microtubes for Standards 1 to 5. Spin down the enclosed 14,15-DHET standard vial (2 μ L, filled with inert gas) and add 1.998 mL of Sample Dilution Buffer to obtain 2 mL of solution. Label this Standard 6. Add 0.1 mL of 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 or N, N-dimethyl-formamide (DMF, 10 μ L to 20 μ L) and vortex well. Before ELISA assay, add 100 μ L 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 the 14,15 EET / DHET 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 μ L 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_0) 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 14,15-DHET-HRP conjugate in the B_0 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 microliters of 2 N sulfuric acid to all of the wells.

Step 11: Read the plate at 450 nm.

CALCULATING THE RESULTS

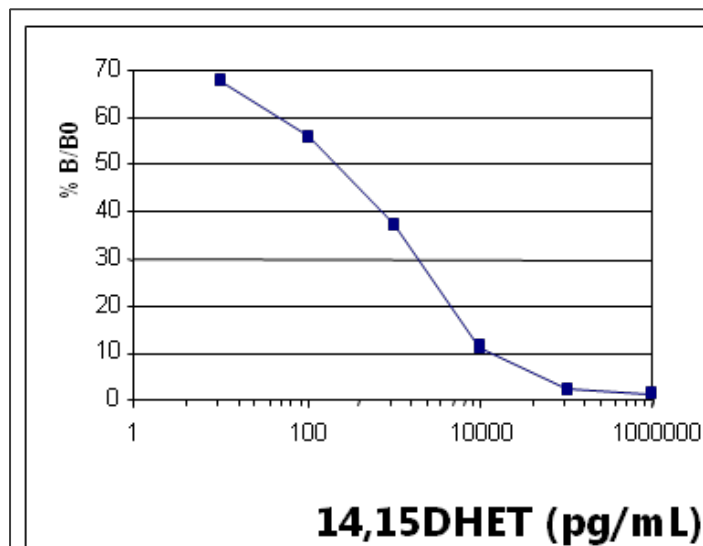
Most plate readers provide data reduction software that can be used to plot the standard curve and determine the sample concentrations. If your plate reader does not have this option, then a data reduction program can be used (4 parameter of log-log curve fit). If you do not have these options, the results 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_0 wells. This is your maximum binding.
3. Calculate the $\%B/B_0$ 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 $\%B/B_0$ versus the concentration of 14,15-DHET from the standards using semi-log paper.
5. Calculate the $\%B/B_0$ for the samples and determine the concentrations, utilizing the standard curve.



- 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 14,15-EET 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 = 0.070
B₀ wells = 0.869

Standard	Concentration	O.D.	%B/B ₀
No. 1	10 pg/mL	0.540	67.6
No. 2	100 pg/mL	0.445	55.7
No. 3	1,000 pg/mL	0.300	37.5
No. 4	10,000 pg/mL	0.094	11.8
No. 5	100,000 pg/mL	0.020	2.5
No. 6	1,000,000 pg/mL	0.011	1.4



Specificity of anti-14,15-DHET IgG

The specificity of the 14,15 EET/DHET ELISA was investigated using authentic 14,15-DHET and a panel of eicosanoids.

Analyte	Specificity
14,15-DHET	100.00%
8,9-DHET	3.30%
11,12-DHET	3.30%
14,15-EET	1.5 %*
15(s) HETE	1.00%
8,9-EET	0.40%
5(s)15(s)DiHETE	0.20%
11,12-EET	0.05%
Arachidonic Acid	0.05%
5,6-DHET	0.02%
5,6-EET	0.02%
Thromboxane B ₂	0.02%
PGE ₂	<0.01 %
PGF _{2a}	<0.01 %
6-keto-PGF _{1a}	<0.01 %

*Recent experiment showed 0.3% cross-reactivity.

REFERENCES

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

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 Information

Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault, or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident.

Eagle Biosciences makes no warranties, either expressed or implied, except as provided herein, including without limitation thereof, warranties as to marketability, merchantability, fitness for a particular purpose or use, or non-infringement of any intellectual property rights. In no event shall the company be liable for any indirect, incidental, or consequential damages of any nature, or losses or expenses resulting from any defective product or the use of any product. Product(s) may not be resold, modified, or altered for resale without prior written approval from Eagle Biosciences, Inc.

For further information about this kit, its application or the procedures in this kit, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.