ab175815 – 11,12 EET / DHET ELISA Kit

Instructions for Use

A competitive immunoenzymatic assay for the quantitative measurement of 11,12 EET / DHET in serum, plasma, urine, cell culture extracts and tissues.

This product is for research use only and is not intended for diagnostic use.

Version 2 Last Updated 17 November 2014

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1. BACKGROUND

Abcam's 11,12 EET / DHET *in vitro* ELISA kit is designed for accurate quantitative measurement in serum, plasma, urine, cell culture, tissue samples following proper isolation and purification.

A 96-well plate has been precoated with 11,12 DHET antibody. Samples and the 11,12 DHET-HRP conjugate are added to the wells, where 11,12 DHETin the sample competes with the added 11,12 DHET-HRP for antibody binding. After incubation, the wells are washed to remove unbound material and TMB substrate is then added which is catalyzed by HRP to produce blue coloration. The reaction is terminated by addition of Stop Solution which stops the color development and produces a color change from blue to yellow. The intensity of signal is inversely proportional to the amount of 11,12 DHET in the sample and the intensity is measured at 450 nm.

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. This kit can be used to measure EET levels in cultured cells which express sEH.

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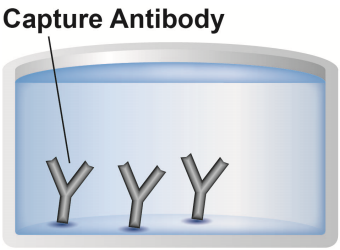
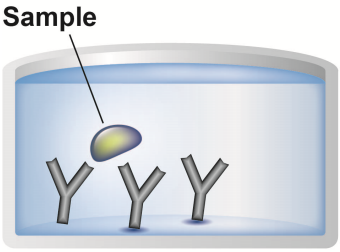
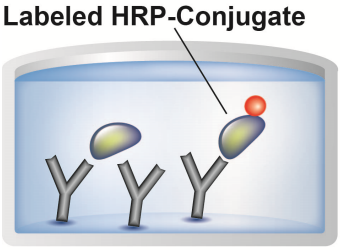
2. ASSAY SUMMARY



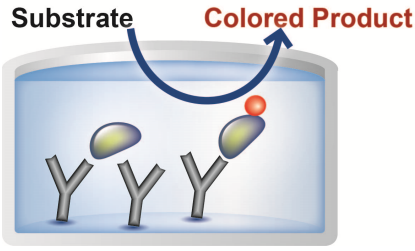
Prepare all reagents and samples as instructed.



Add standards and samples to each well used.



Add prepared HRP conjugate to each well and incubate at room temp.



Add TMB substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at 2-8°C or 20°C immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 9. Reagent Preparation.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (After Preparation)
11,12 DHET ELISA plate	96 Wells	2-8°C
11,12 DHET Standard (1 mg/mL)	2 µL	2-8°C
1,000X 11,12 DHET HRP Conjugates	12 µL	2-8°C
10X Sample Dilution Buffer	25 mL	2-8°C
HRP Buffer	15 mL	2-8°C
10X Wash Buffer Solution	25 mL	2-8°C
TMB Substrate	24 mL	2-8°C

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Microplate reader capable of measuring absorbance at 450 nm
- Incubator at 37°C
- Multi- and single-channel pipettes to deliver volumes between 10 and 1,000 µL
- Optional: Automaticplate washer for rinsing wells.
- Storage bottles
- Rotating mixer
- Deionised or (freshly) distilled water.
- Disposable tubes
- Timer
- 2N Sulfuric acid

7. LIMITATIONS

- ELISA kit intended for research use only. Not for use in diagnostic procedures
- Use only clean pipette tips, dispensers, and lab ware
- Do not interchange screw caps of reagent vials to avoid cross-contamination
- Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination
- After first opening and subsequent storage check conjugate and control vials for microbial contamination prior to further use
- To avoid cross-contamination and falsely elevated results pipette patient samples and dispense conjugate, without splashing, accurately to the bottom of wells

8. TECHNICAL HINTS

- Avoid foaming or bubbles when mixing or reconstituting components
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions
- Ensure plates are properly sealed or covered during incubation steps
- Complete removal of all solutions and buffers during wash steps is necessary for accurate measurement readings
- Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction
- It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate
- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

9. REAGENT PREPARATION

Equilibrate all reagents, samples and controls to room temperature (18-25°C) prior to use.

9.1 1X Wash Buffer

Mix the 10X Wash Buffer Solution with a stir bar, applying low, gentle heat until a clear colorless solution is obtained. Dilute the entire contents of the 10X Wash Buffer Solution (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.

9.2 1X HRP Conjugate

Dilute 1 vial of the 11,12 DHET-HRP conjugate (12 µL) with 12 mL of 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.

9.3 1X Sample Dilution Buffer

Prepare 1X Sample Dilution Buffer by adding 25 mL of 10X Sample Dilution Buffer to 225 mL of dH_20. Mix gently and thoroughly.

10. STANDARD PREPARATION

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of positive controls for every use.

10.1 Label 5 microtubes as Standard # 2 - 6.

10.2 Add 900 µL of the 1X Sample Dilution Buffer to the microtubes for Standards # 2 to 6.

10.3 Prepare a 1 µg/mL **Standard #1** by first spinning down the enclosed 11,12 DHET standard vial (2 µL, filled with inert gas) and then adding 1.998 mL of 1X Sample Dilution Buffer to obtain 2 mL of solution.

10.4 Prepare **Standard #2** by adding 100 µL of the Standard #1 to the microtube labeled **Standard #2**. Mix thoroughly and gently.

10.5 Prepare **Standard #3** by adding 100 µL of the Standard #2 to the microtube labeled **Standard #3**. Mix thoroughly and gently.

10.6 Using the table below as a guide, repeat for tubes #4 through #6.

10.7 Standard B_o contains no protein and is blank control.

Standard #	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	Step 10.3				1,000,000
2	Standard #1	100	900	1,000,000	100,000
3	Standard #2	100	900	100,000	10,000
4	Standard #3	100	900	10,000	1,000
5	Standard #4	100	900	1,000	100
6	Standard #5	100	900	100	10
B_o	None		900	-	-

11. SAMPLE COLLECTION AND STORAGE

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 P_{450} 2C23 activity of the rat microsomes was measured, the rat microsomes were incubated with arachidonic acid (substrate of P_{450} 2C23) and, then, EET + DHET levels in the reaction mixture were measured after acid hydrolysis of EET to DHET, which was indicative of P_{450} 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.

11.1 Protocol #1: EET formation activity measurement

11.1.1 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.

11.1.2 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.)

11.1.3 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.

11.1.4 Evaporate the pooled ethyl acetate until all is dried up under argon gas.

11.1.5 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 Dilution Buffer to three wells.)

11.1.6 Perform the ELISA for 11,12 DHET

11.2 Protocol #2: Free EEt + DHET formation activity measurement

11.2.1 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.

11.2.2 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.

11.2.3 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.

11.2.4 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.

11.2.5 For ELISA assay, dissolve the sediment in 20 µL of ethanol or DMF (vortex thoroughly), then add 130 µL 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).

11.2.6 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.

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

11.3.1 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.

11.3.2 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.

11.3.3 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)].

11.3.4 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.

11.3.5 Dissolve the dried residue in a minimal amount of ethanol (~20 µL), 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.

11.3.6 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.

11.3.7 For ELISA, dissolve the sediment in 20 µL of ethanol or DMF (vortex thoroughly), then add 130µL 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).

11.3.8 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. Subtract that value from the EET + DHET level and you will obtain the EET level in the sample

12. PLATE PREPARATION

- The 96 well plate included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents
- Unused well strips should be returned to the plate packet and stored at 4°C
- For each assay performed, a minimum of 2 wells must be used as a blank, omitting sample and conjugate from well addition. Another 2 wells must be used for a maximum binding control
- For statistical reasons, we recommend each standard and sample should be assayed with a minimum of two replicates (duplicates)

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use**
- **Please read the test protocol carefully before performing the assay. Result reliability depends on strict adherence to the test protocol as described**
- **If performing the test on anautomatic ELISA system we recommend increasing the washing steps from three to five and the volume of 1X Wash Bufferfrom 300 µL to 350 µL to avoid washing effects**
- **Assay all standards, controls and samples in duplicate**

13.1 Add 200 µL of 1X Sample Dilution Buffer into the blank wells and 100 µL of1XSample Dilution Buffer into maximum binding control wells.

13.2 Add 100 µL of each of the standards or samples into the appropriate wells.

13.3 Add 100 µL of the 1X-HRP conjugate in the all wells except the blank control wells.

13.4 Incubate the plate at room temperature for two hours.

13.5 Wash the plate three times with 400 µL of 1X Wash Buffer per well.

13.6 After the last of the three wash cycles pat the inverted plate dry onto some paper towels.

13.7 Add 200 µL of the TMB substrate to all of the wells.

13.8 Incubate the plate at room temperature for 15-30 minutes.

13.9 Add 50 µL of 2 N sulfuric acid to all of the wells.

13.10 Read the plate at 450 nm.

14. CALCULATIONS

If data redaction software is not available on your plate reader then the results can be obtained manually as follows:

14.1 Average the absorbance (Abs) readings from the blank wellsand 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.)

14.2 Average the corrected absorbance readings from the maximum binding control wells. This is your maximum binding.

14.3 Calculate the % Abs for Standard 1 by averaging the corrected absorbance of the two wells; divide the average by the Maximum Binding Control well average absorbance, then multiply by 100. Repeat this formula for the remaining standards.

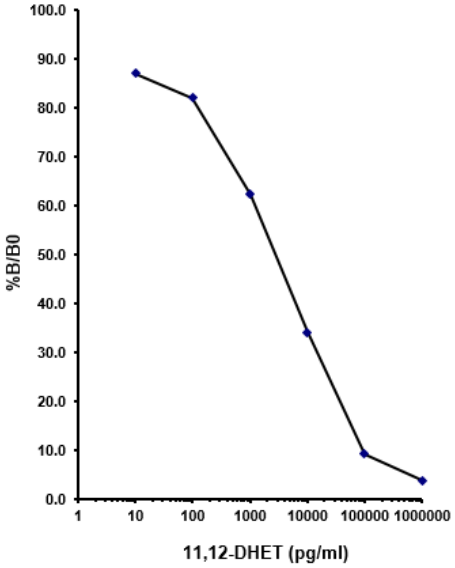
14.4 Plot the % Abs versus the concentration of 11,12 DHET from the standards using semi-log paper.

14.5 Calculate the % Abs for the samples and determine the concentrations, utilizing the standard curve.

14.6 Multiply the concentrations obtained for each of the samples by their corresponding dilution factor.

15. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.The data shown here is an example of typical results obtained using the Abcam's11,12 DHET ELISA kit. These results are only a guideline, and should not be used to determine values from your samples.



Conc. (pg/mL)	% (B/B_0)
10	87.0
100	82.0
1,000	62.3
10,000	34.1
100,000	9.3
1,000,000	3.9

16. ASSAY SENSITIVITY

Target		Intra-Assay	Inter-Assay
11,12 EET	n=	12	12
	CV (%)	0.210	1.820

SENSITIVITY –

The calculated minimal detectable (MDD) dose is 10 pg/mL. The MDD was determined by calculating the mean of zero standard replicates.

17. TROUBLESHOOTING

Problem	Cause	Solution
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	The TMB substrate was not added	Add substrate
	The TMB conjugate was not incubated for the proper time	Continue incubation until desired color is reached

Problem	Cause	Solution
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 OD obtained from sample	Incorrect loading of samples	Redo assay using an 8 channel pipetman making sure the 8 channels are equal volume while loading

18. NOTES

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