コスモ・バイオ株式会社

11,12-DHET

ELISA KIT

Detroit R&D, Inc.

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Rev. date: 4/26/2005

To Order Call: (313)961-1606

コスモ・バイオ株式会社

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TROUBLE SHOOTING

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 tempera ture.
- The lab is to 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 same sample

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

Table of Conten

KIT CONTENTS

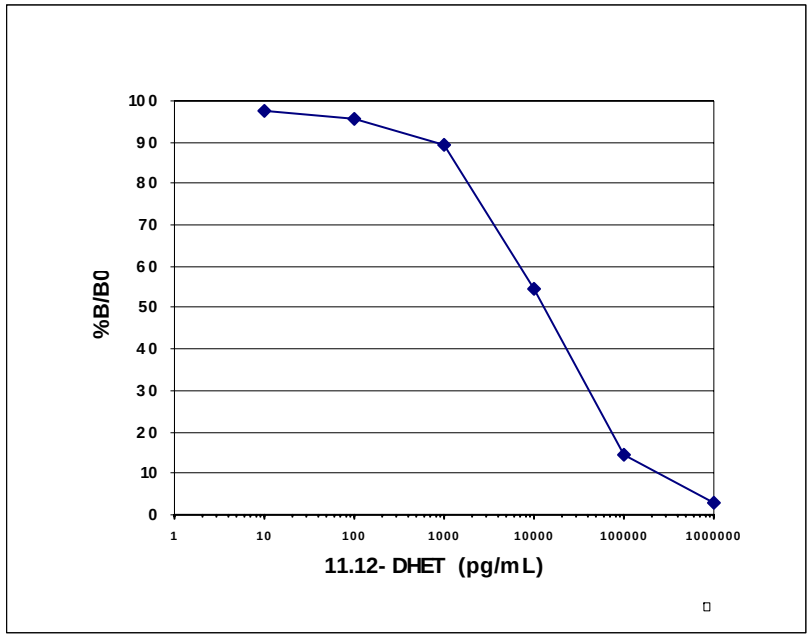
Part Number	Item	Description	Quantity
1	11,12-DHET ELISA Plate	Solid 96-well plate coated with anti-11,12-DHET antibody per well	1
2	11,12-DHET Standard (2 µl)	Stock standard at a concentration of 1 mg/ml	1
3	11,12-DHET-HRP Conjugates (12 µl)	1000 X concentrated solution	1
4	Sample Dilution Stock Buffer (25 ml)	A 10 X solution of Tris-buffered saline with preservatives	1
5	HRP Dilution Buffer (30 ml)	A concentrated solution of Tris-buffered saline with preservatives	1
6	Wash Buffer Stock Solution (50 ml)	A 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

NOTES:

Production of 11,12-DHET EL

Anti-11,12-DHET IgG was isol om goat anti-11,12-DHET sera using protein G affinity column c ography. The goat was immunized with 11,12-DHET conjugated t *via* carboxyl group of the 11,12-DHET. Specificity of the 11,12 IgG was assayed with authentic eicosanoids structurally similar to 11,12-DHET.

Typical Results



The data shown here is an example of typical results obtained using the Detroit R & D 11,12-DHET 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.

Blank wells = 0.089
B_0 wells = 0.1.03

Standard	Concentration	O.D.	% B/B_0
No. 1	10 pg/ml	1.004	97.8
No. 2	100 pg/ml	0.986	95.8
No. 3	1,000 pg/ml	0.927	89.5
No. 4	10,000 pg/ml	0.599	54.5
No. 5	100,000 pg/ml	0.224	14.4
No. 6	1000,000 pg/ml	0.118	3.03

PRECAUTIONS

- Please read these instru carefully before beginning this 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

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

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 at –20 °C and should not be re-frozen and thawed any more than is necessary.

Additional Items Required

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

Background Information

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 EET's, which are generated by arachidonic acid epoxygenase activity of cytochrome P450's[4].

This is a competitive ELISA kit, based on competition between 11,12-DHET epitope and 11,12-DHET-HRP conjugate for 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-DHET in 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 in the conjugate forming a blue color in the well, which will be more or less intense based upon the amount of 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.

References

[1] Kim et al. Two divisional US Patents: 6,440,682 and 6,534,282, issued on 8/27/2002 & 3/18/2003, respectively. (http://patft.uspto.gov/netahtml/srchnum.htm

Pat.US: 6,440,682 & Pat.US: 6,534,282)

[2] Sinal et al. Targeted disruption of soluble epoxide hydrolase reveals a role in blood pressure regulation. J. Biol. Chem. 275, 40504, 2000.

[3] Yu et al. Soluble epoxide hydrolase regulates hydrolysis of vasoactive epoxyeicosatrienoic acids. Circulation Research 87, 992, 2000.

[4] 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.

* Please contact us for urine or plasma extraction procedure or human study.

5) Calculate the %B/B_0 for th les and determine the concentrations, utilizing the st curve.

6) Multiply the concentrations ıed for each of the samples by their corresponding dilutior .

Calculations

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 obtain 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 users manual for 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 S1 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 11,12-DHET from the standards using semi–log paper.

Assay P arations

- **Note:** Remove all of the re required, including the TMB, and allow them to equilib room temperature before proceeding. It is necessary roughly mix the concentrated buffer . A stir bar is cont within each buffer solution.

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 (50 ml) with 450 ml of deionized water to yield a final volume of 500 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 11.88 ml of HRP dilution buffer to yield a final volume of 12 ml. 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 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.

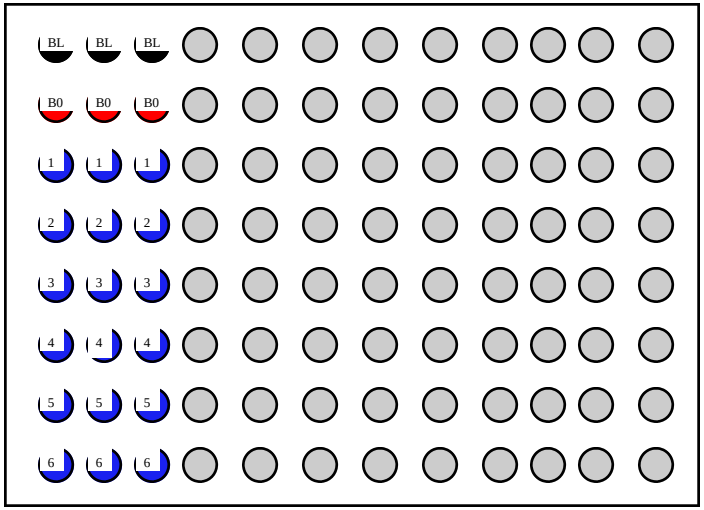
SAMPLES

Samples can be directly diluted into the 1 X Sample Dilution Buffer. A 1:10 dilution should remove most interfering substances which may be in the sample. If a rough estimate of the concentration of 11,12-DHET is not known, it is recommended to dilute the sample at magnitudes of 10 (1:10, 1:100, 1:1000 etc.) until the results fall into the readable portion of the standard curve (between 10 ng/ml and 0.01 ng/ml).

Performing The Assay

Plate Setup

Each plate must contain a minimum of three blank wells (BL), three maximum binding wells (B_0), and a six point standard curve (S1-S6). Each sample should be assayed in triplicate. A suggested plate format is shown as follows:



Standards Dilution Table

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

Step 1) Load 100 microliters ple Dilution Buffer into the maximum binding (B and the blank (BL) wells.

Step 2) Load 100 microliters ı of the standards into the appropriate wells.

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

Step 4) Load 100 microliters of the diluted 11,12-DHET -HRP conjugate into the B_0 wells, the standard wells, and the sample wells. Do NOT add HRP conjugate into blank (BL) wells.

Step 5) Incubate the plate at room temperature for 2 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 BL (blank wells).

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