11-dehydro Thromboxane B_2 ELISA Kit - Monoclonal

Item No. 519510

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size	480 wells Quantity/Size
419512	11-dehydro Thromboxane B_2 Monoclonal Antibody	1 vial/100 dtn	1 vial/500 dtn
419510	11-dehydro Thromboxane B_2 Monoclonal AChE Tracer	1 vial/100 dtn	1 vial/500 dtn
419514	11-dehydro Thromboxane B_2 Monoclonal ELISA Standard	1 vial	1 vial
419517	11-dehydro Thromboxane B_2 Monoclonal Assay Buffer Concentrate (10X)	2 vials/10 ml	4 vials/10 ml
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	1 vial/12.5 ml
400035	Polysorbate 20	1 vial/3 ml	1 vial/3 ml
400009/400008	Goat Anti-Mouse IgG Coated Plate	1 plate	5 plates
400012	96-Well Cover Sheet	1 cover	5 covers
400050	Ellman's Reagent	3 vials/100 dtn	6 vials/250 dtn
400040	ELISA Tracer Dye	1 vial	1 vial
400042	ELISA Antiserum Dye	1 vial	1 vial

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's AChE ELISA Kits. This kit may not perform as described if any reagent or procedure is replaced or modified.

When compared to quantification by LC/MS or GC/MS, it is not uncommon for immunoassays to report higher analyte concentrations. While LC/MS or GC/MS analyses typically measure only a single compound, antibodies used in immunoassays sometimes recognize not only the target molecule, but also structurally related molecules, including biologically relevant metabolites. In many cases, measurement of both the parent molecule and metabolites is more representative of the overall biological response than is the measurement of a short-lived parent molecule. It is the responsibility of the researcher to understand the limits of both assay systems and to interpret their data accordingly.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance between 405-420 nm.
2. An adjustable pipettor.
3. A source of 'UltraPure' water. Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*
4. Materials used for **Sample Preparation** (see page 13).

INTRODUCTION

Background

Thromboxane A_2 (TXA_2) is produced from arachidonic acid and causes irreversible platelet aggregation, and contraction of both vascular and bronchial smooth muscle.[1-3] TXA_2 is rapidly hydrolyzed non-enzymatically to form TXB_2. Although it is common to estimate TXA_2 levels by measuring TXB_2, most of the TXB_2 measured in the plasma or urine of healthy individuals is due to *ex vivo* platelet activation or intra-renal production, respectively.[4] Measurement errors are compounded by the fact that normal concentrations of circulating TXB_2 are extremely low (1-2 pg/ml) and highly transient ($t_{1/2}$ = 5-7 minutes).[5-6]

To circumvent this problem, it is necessary to measure a metabolite that cannot be formed by platelets or by the kidney. TXB_2 can be metabolized by one or more 11-dehydro-TX dehydrogenase enzymes (including the cytosolic aldehyde dehydrogenase (EC1.2.1.3)) to form 11-dehydro TXB_2 or by peroxisomal β-oxidation to form 2,3,-dinorTXB_2.[7-11] Infusion studies using TXB_2 have shown that although both metabolites are formed, 11-dehyro TXB_2 has a longer circulating half-life ($t_{1/2}$ = 45 minutes) than does 2,3-dinor TXB_2 ($t_{1/2}$ = 15 minutes).[6,12] Therefore, measurement of 11-dehydro TXB_2 in plasma or urine will give a time-integrated indication of TXA_2 production.

Although 11-dehydro TXB_2 can be found in substantial amounts in urine, this compound is also subject to further metabolism, including β-oxidation. β-oxidation of 11-dehydro TXB_2 results in the formation of 11-dehydro-2,3-dinor TXB_2, which is also found in substantial quantities in urine.[7,13] Djurup, *et al*. demonstrated that an immunoassay that measures the sum of 11-dehydro TXB_2 and 11-dehydro-2,3-dinor TXB_2 correlated well with measurement of 11-dehydro TXB_2 alone by GC/MS in urine samples from healthy subjects before and after dosing with aspirin.[14] Studies in smokers have also demonstrated that changes in 11-dehydro-2,3-dinor TXB_2 parallel changes in 11-dehydro TXB_2.[15] This kit measures both 11-dehydro TXB_2 and 11-dehydro-2,3-dinor TXB_2.

Normal plasma levels of 11-dehydro TXB_2 (1-2 pg/ml) are below the detection limit of this assay, so plasma samples need to be purified and concentrated prior to assay. There is evidence suggesting that 11-dehydro TXB_2 can be formed non-enzymatically following platelet activation, so it is important to process plasma samples quickly to prevent artifactual formation of 11-dehydro TXB_2.[16,17]

About This Assay

Cayman's 11-dehydro TXB_2 ELISA Kit is a competitive assay that can be used for quantification of 11-dehydro TXB_2 in urine and other sample matrices. The assay has a range from 15.6-2,000 pg/ml and a sensitivity (80% B/B_0) of approximately 34 pg/ml.

Description of AChE Competitive ELISAs[18, 19]

This assay is based on the competition between free 11-dehydro TXB_2 and an 11-dehydro TXB_2-acetylcholinesterase (AChE) conjugate (tracer) for a limited number of 11-dehydro TXB_2-specific Monoclonal Antibody binding sites. The concentration of the tracer is held constant while the concentration of free 11-dehydro TXB_2 varies. Thus, the amount of tracer that is able to bind to the 11-dehydro TXB_2 Monoclonal Antibody will be inversely proportional to the concentration of free 11-dehydro TXB_2 in the well. The Monoclonal Antibody-11-dehydro TXB_2 complex (either free or tracer) binds to the goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of tracer bound to the well, which is inversely proportional to the amount of free 11-dehydro TXB_2 present in the well during the incubation; or

Absorbance $\propto$ [Bound 11-dehydro TXB_2 Tracer] $\propto$ 1/[11-dehydro TXB_2]

A schematic of this process is shown in Figure 1, below.

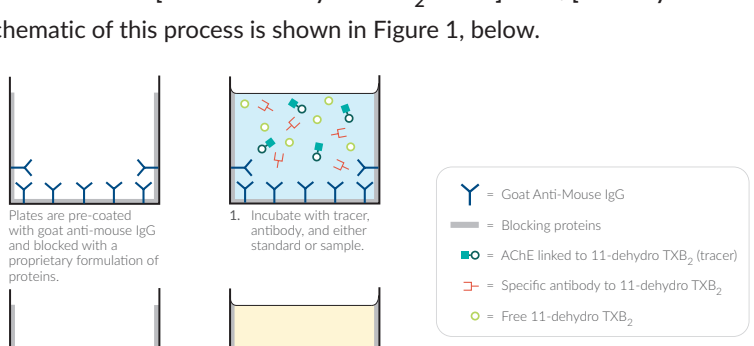


Figure 1. Schematic of the AChE ELISA

Biochemistry of Acetylcholinesterase

The electric organ of the electric eel, *E. electricus*, contains an avid AChE capable of massive catalytic turnover during the generation of its electrochemical discharges. The electric eel AChE has a clover leaf-shaped tertiary structure consisting of a triad of tetramers attached to a collagen-like structural fibril. This stable enzyme is capable of high turnover (64,000 s^{-1}) for the hydrolysis of acetylthiocholine.

A molecule of the analyte covalently attached to a molecule of AChE serves as the tracer in AChE enzyme immunoassays. Quantification of the tracer is achieved by measuring its AChE activity with Ellman's Reagent. This reagent consists of acetylthiocholine and 5,5'-dithio-*bis*-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine (see Figure 2, on page 10). The non-enzymatic reaction of thiocholine with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm (ε = 13,600).

AChE has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. This property of AChE also allows redevelopment of the assay if it is accidentally splashed or spilled. In addition, the enzyme is highly stable under the assay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts or preservatives. Since AChE is stable during the development step, it is unnecessary to use a 'stop' reagent, and the plate may be read whenever it is convenient.

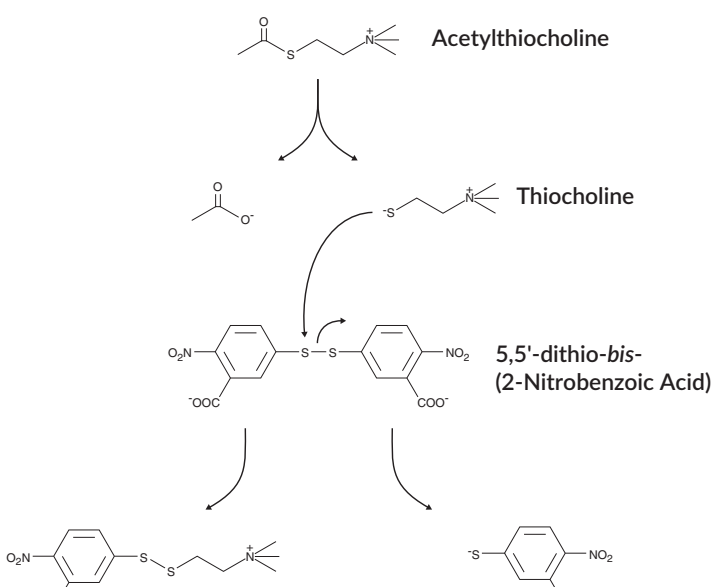


Figure 2. Reaction catalyzed by acetylcholinesterase

Definition of Key Terms

Blank: background absorbance caused by Ellman's Reagent. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including NSB wells.

Total Activity: total enzymatic activity of the AChE-linked tracer. This is analogous to the specific activity of a radioactive tracer.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding. Do not forget to subtract the Blank absorbance values.

B_0 (Maximum Binding): maximum amount of the tracer that the antibody can bind in the absence of free analyte.

%B/B_0 (%Bound/Maximum Bound): ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B_0) well.

Standard Curve: a plot of the %B/B_0 values *versus* concentration of a series of wells containing various known amounts of analyte.

Dtn: determination, where one dtn is the amount of reagent used per well.

Cross Reactivity: numerical representation of the relative reactivity of this assay towards structurally related molecules as compared to the primary analyte of interest. Biomolecules that possess similar epitopes to the analyte can compete with the assay tracer for binding to the primary antibody. Substances that are superior to the analyte in displacing the tracer result in a cross reactivity that is greater than 100%. Substances that are inferior to the primary analyte in displacing the tracer result in a cross reactivity that is less than 100%. Cross reactivity is calculated by comparing the mid-point (50% B/B_0) value of the tested molecule to the mid-point (50% B/B_0) value of the primary analyte when each is measured in assay buffer using the following formula:

$$\text{\% Cross Reactivity} = \left[\frac{\text{50\% } B/B_0 \text{ value for the primary analyte}}{\text{50\% } B/B_0 \text{ value for the potential cross reactant}} \right] \text{x } 100\%$$

PRE-ASSAY PREPARATION

NOTE: Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA. UltraPure water may be purchased from Cayman (Item No. 400000).

Buffer Preparation

1. **11-dehydro TXB_2 Monoclonal Assay Buffer Concentrate (10X) Preparation**

   Dilute the contents of one vial of 11-dehydro TXB_2 Monoclonal Assay Buffer Concentrate (10X) (Item No. 419517) with 90 ml of UltraPure water. Be certain to rinse the vial to remove any salts that may have precipitated. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.*

   Store diluted buffer at 4°C. It should be stable for at least two weeks.

2. **Wash Buffer Preparation**

   5 ml vial Wash Buffer Concentrate (400X) (96-well kit; Item No. 400062): Dilute to a total volume of 2 liters with UltraPure water and add 1 ml of Polysorbate 20 (Item No. 400035).

   OR

   12.5 ml vial Wash Buffer Concentrate (400X) (480-well kit; Item No. 400062): Dilute to a total volume of 5 liters with UltraPure water and add 2.5 ml of Polysorbate 20 (Item No. 400035).

   Store diluted buffer at 4°C. It should be stable for approximately two months.

Smaller volumes of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Polysorbate 20 (0.5 ml/liter of Wash Buffer).

NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.

Sample Preparation

This assay has been validated for urine. Other sample types may also be used, but the purification protocols have not been fully tested.

Proper sample storage and preparation are essential for consistent and accurate results. Please read this section carefully before beginning the assay. *NOTE: 11-dehydro TXB_2 is capable of existing in two different conformations. The 11-dehydro TXB_2 Monoclonal Assay Buffer supplied with this kit is designed to convert all of the 11-dehydro TXB_2 into one conformation for more consistent results. All standards and samples should be diluted using this buffer (unpurified samples should be diluted at least 1:2), and incubated at room temperature for at least two hours before beginning the assay. Overnight incubation, if more convenient, is acceptable.*

General Precautions

- All samples must be free of organic solvents prior to assay.
- Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C.
- Samples of mouse or rat origin may contain antibodies which interfere with the assay by binding to the goat anti-mouse IgG-coated plate.

Testing for Interference

Urine, plasma, and serum, as well as other heterogeneous mixtures, such as lavage fluids and aspirates, often contain contaminants which can interfere in immunoassays. It is best to check for interference before embarking on a large number of sample measurements. To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between approximately 50 and 500 pg/ml (*i.e.*, between 20-80% B/B_0). If the two different dilutions of the sample show good correlation (differ by 20% or less) in the final calculated 11-dehydro TXB_2 concentration, purification is not required. If you do not see good correlation of the different dilutions, purification is advised. As described above, samples which are not purified should be diluted with 11-dehydro TXB_2 Monoclonal Assay Buffer and incubated at room temperature for at least two hours before beginning the assay to convert all of the 11-dehydro TXB_2 in the sample to the same conformation as the standards.

Urine

Incubate urine samples briefly on ice and centrifuge to remove precipitated proteins. Dilutions of urine of 1:2 or greater show a direct linear correlation between 11-dehydro TXB_2 immunoreactivity and 11-dehydro TXB_2 concentration. As with any urinary marker, we recommend standardizing the values obtained by EIA to creatinine levels (Item No. 500701).

Plasma

NOTE: This procedure has not been fully validated for plasma samples. Plasma should be collected in vacutainers containing sodium heparin, EDTA, or sodium citrate. Vacutainers should be supplemented with indomethacin to give a final concentration of at least 10 µM. Indomethacin will prevent *ex vivo* formation of eicosanoids, which have the potential to interfere with this assay, although most eicosanoids do not appear to exhibit any cross reactivity (see below). There is evidence suggesting that 11-dehydro TXB_2 can be formed non-enzymatically following platelet activation, so it is important to process plasma samples quickly to prevent artifactual formation of 11-dehydro TXB_2.[16,17]

Normal plasma levels of 11-dehydro TXB_2 (1-2 pg/ml) are below the limit of detection of this assay. However, pathological conditions in which platelets become activated (*e.g.* unstable angina) can result in plasma levels of 11-dehydro TXB_2 that are sufficiently elevated to measure with this assay.[20,21] Acetone precipitation (see page 16) is recommended as a preparative step for plasma sample analysis.

Processing Plasma-Acetone Precipitation

Thaw plasma samples. Immediately add four volumes of ice cold acetone and vortex briefly. Incubate for 15 minutes at 4°C and centrifuge at 5,000 x g. Transfer the supernatant into a clean test tube. Dry this acetone mixture under a stream of nitrogen, and reconstitute with a volume of 11-dehydro TXB_2 Monoclonal Assay Buffer equal to the original plasma volume. If you wish to concentrate your samples, resuspend them in a volume of EIA Buffer that is smaller than the original volume. Incubate for two hours at room temp or overnight at 4°C (depending on the standard incubation preference).

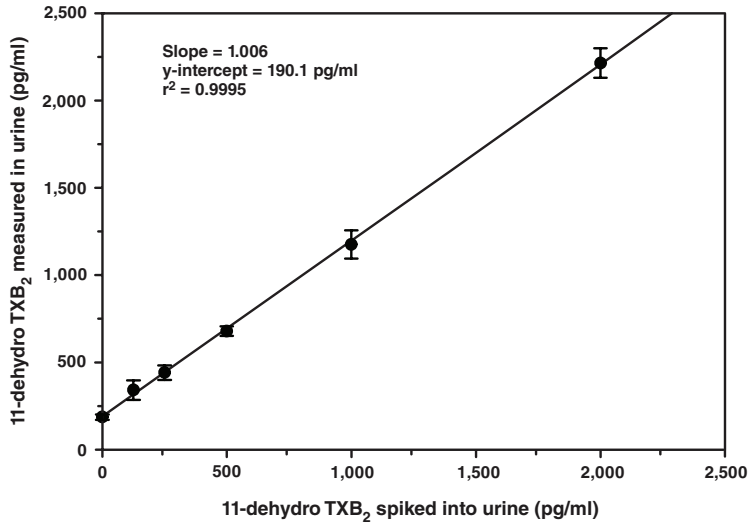


Figure 3. Recovery of 11-dehydro TXB_2 from urine. Urine samples were spiked with 11-dehydro TXB_2, diluted as described in the **Sample Preparation** section and analyzed using this kit. The y-intercept corresponds to the amount of 11-dehydro TXB_2 in unspiked urine. Error bars represent standard deviations obtained from multiple dilutions of each sample.

Level	%CV Intra-assay variation	Average (pg/ml)	%CV Inter-assay variation	Average (pg/ml)
High	5.0	465	6.0	453
Medium	7.4	196	12.9	175
Low	7.5	77	11.7	69

Table 1. Urine sample validation

Urine samples containing a high, medium, or low level of 11-dehydro TXB_2 were measured 56 times each using a single set of reagents. The calculated %CV is reported as intra-assay variance. A separate series of urine samples containing a high, medium, or low level of 11-dehydro TXB_2 were measured four times each using eight independent sets of reagents. The calculated %CV is reported as inter-assay variance.

Preparation of Assay-Specific Reagents

11-dehydro TXB_2 Monoclonal ELISA Standard

Equilibrate a pipette tip in ethanol by repeatedly filling and expelling the tip with ethanol. Using the equilibrated pipette tip, transfer 100 µl of the 11-dehydro TXB_2 Monoclonal ELISA Standard (Item No. 419514) into a clean test tube, then dilute with 900 µl of UltraPure water. The concentration of this solution (the bulk standard) will be 20 ng/ml. Store this solution at 4°C; it will be stable for approximately six weeks.

To prepare the standard for use in ELISA: Obtain eight clean test tubes and number them #1 through #8. Aliquot 900 µl of 11-dehydro TXB_2 Monoclonal Assay Buffer to tube #1 and 500 µl of 11-dehydro TXB_2 Monoclonal Assay Buffer to tubes #2-8. Transfer 100 µl of the bulk standard (20 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place into tube #3; mix thoroughly. Repeat this process for tubes #4-8. Incubate these standards at room temperature for a minimum of two hours before adding to the plate. Alternatively the standards and samples can be diluted with 11-dehydro TXB_2 Monoclonal Assay Buffer and incubated overnight at 4°C. On the following day, allow all reagents to equilibrate to room temperature prior to use and follow the standard procedure described on page 25 in the **Performing the Assay** section. The diluted standards may be stored at 4°C for no more than 24 hours.

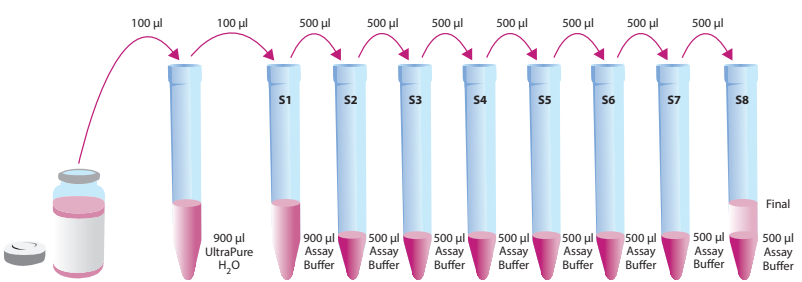


Figure 4. Preparation of the 11-dehydro TXB_2 standards

11-dehydro TXB_2 Monoclonal AChE Tracer

Reconstitute the 11-dehydro TXB_2 Monoclonal AChE Tracer as follows:

100 dtn 11-dehydro TXB_2 Monoclonal AChE Tracer (96-well kit; Item No. 419500): Reconstitute with 6 ml 11-dehydro TXB_2 Monoclonal Assay Buffer.

OR

500 dtn 11-dehydro TXB_2 Monoclonal AChE Tracer (480-well kit; Item No. 419500): Reconstitute with 30 ml 11-dehydro TXB_2 Monoclonal Assay Buffer.

The tracer may be used immediately following reconstitution. Store the reconstituted 11-dehydro TXB_2 Monoclonal AChE Tracer at 4°C *(do not freeze!)* and use within two weeks. A 20% surplus of tracer has been included to account for any incidental losses.

Tracer Dye Instructions (optional)

This dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the reconstituted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer or add 300 µl of dye to 30 ml of tracer).

11-dehydro TXB_2 Monoclonal Antibody

Reconstitute the 11-dehydro TXB_2 Monoclonal Antibody as follows:

100 dtn 11-dehydro TXB_2 Monoclonal Antibody (96-well kit; Item No. 419502): Reconstitute with 6 ml 11-dehydro TXB_2 Monoclonal Assay Buffer.

OR

500 dtn 11-dehydro TXB_2 Monoclonal Antibody (480-well kit; Item No. 419502): Reconstitute with 30 ml 11-dehydro TXB_2 Monoclonal Assay Buffer.

The antibody may be used immediately following reconstitution. Store the reconstituted 11-dehydro TXB_2 Monoclonal Antibody at 4°C. It will be stable for at least two weeks. A 20% surplus of antibody has been included to account for any incidental losses.

Antiserum Dye Instructions (optional)

This dye may be added to the antibody, if desired, to aid in visualization of antibody-containing wells. Add the dye to the reconstituted antibody at a final dilution of 1:100 (add 60 µl of dye to 6 ml antibody or add 300 µl of dye to 30 ml of antibody).

Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. *NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

Each plate or set of strips must contain a minimum of two blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B_0), and an eight point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* Each sample should be assayed at two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown in Figure 5, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 25, for more details). We suggest you record the contents of each well on the template sheet provided (see page 34).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	S1	S1	1	1	1	9	9	9	17	17	17
B	Blk	S2	S2	2	2	2	10	10	10	18	18	18
C	NSB	S3	S3	3	3	3	11	11	11	19	19	19
D	NSB	S4	S4	4	4	4	12	12	12	20	20	20
E	B_0	S5	S5	5	5	5	13	13	13	21	21	21
F	B_0	S6	S6	6	6	6	14	14	14	22	22	22
G	B_0	S7	S7	7	7	7	15	15	15	23	23	23
H	TA	S8	S8	8	8	8	16	16	16	24	24	24

Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8
1-24 - Samples

Figure 5. Sample plate format

Performing the Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Addition of the Reagents

1. **11-dehydro TXB_2 Monoclonal Assay Buffer**

   Add 100 µl 11-dehydro TXB_2 Monoclonal Assay Buffer to NSB wells. Add 50 µl 11-dehydro TXB_2 Monoclonal Assay Buffer to B_0 wells.

2. **11-dehydro TXB_2 Monoclonal EIA Standard**

   After the standards have been diluted and incubated at room temperature for at least two hours (or optional overnight 4°C incubation) they may be added to the plate. Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in the standard.

3. **Samples**

   Dilute samples a minimum of 1:2 with 11-dehydro TXB_2 Monoclonal Assay Buffer, incubate at room temperature for at least two hours (or optional overnight 4°C incubation) add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **11-dehydro TXB_2 Monoclonal AChE Tracer**

   Add 50 µl to each well *except* the TA and the Blk wells.

5. **11-dehydro TXB_2 Monoclonal Antibody**

   Add 50 µl to each well *except* the TA, the NSB, and the Blk wells.

Well	Assay Buffer	Standard/Sample	Tracer	Antibody
Blk	-	-	-	-
TA	-	-	5 µl (at devl. step)	-
NSB	100 µl	-	50 µl	-
B_0	50 µl	-	50 µl	50 µl
Std/Sample	-	50 µl	50 µl	50 µl

Table 1. Pipetting summary

Incubation of the Plate

Cover each plate with plastic film (Item No. 400012) and incubate for two hours at room temperature on an orbital shaker.

Development of the Plate

1. Reconstitute Ellman's Reagent immediately before use (20 ml of reagent is sufficient to develop 100 wells):

   100 dtn vial Ellman's Reagent (96-well kit; Item No. 400050): Reconstitute with 20 ml of UltraPure water.

   OR

   250 dtn vial Ellman's Reagent (480-well kit; Item No. 400050): Reconstitute with 50 ml of UltraPure water.

NOTE: Reconstituted Ellman's Reagent is unstable and should be used the same day it is prepared; protect the Ellman's Reagent from light when not in use. Extra vials of the reagent have been provided should a plate need to be re-developed or multiple assays be run on different days.

2. Empty the wells and rinse five times with Wash Buffer.
3. Add 200 µl of Ellman's Reagent to each well.
4. Dilute the 11-dehydro TXB_2 Monoclonal AChE Tracer 1:10 with 11-dehydro TXB_2 Monoclonal Assay Buffer (for example, 50 µl Tracer into 450 µl Assay Buffer).
5. Add 5 µl of tracer to the TA wells.
6. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark. This assay typically develops (*i.e.*, B_0 wells ≥0.3 A.U. (blank subtracted)) in 60-90 minutes.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Remove the plate cover being careful to keep Ellman's Reagent from splashing on the cover. *NOTE: Any loss of Ellman's Reagent will affect the absorbance readings. If Ellman's Reagent is present on the cover, use a pipette to transfer the Ellman's Reagent into the well. If too much Ellman's Reagent has splashed on the cover to easily redistribute back into the wells, wash the plate three times with wash buffer and repeat the development with fresh Ellman's Reagent.*
3. Read the plate at a wavelength between 405 and 420 nm (usually 412 nm). The absorbance may be checked periodically until the B_0 wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B_0 wells in the range of 0.3-1.5 A.U. (blank subtracted). If the absorbance of the wells exceeds 1.5, wash the plate, add fresh Ellman's Reagent and let it develop again.

ANALYSIS

Many plate readers come with data reduction software that plots data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as either $\%B/B_0$ *versus* log concentration using a four-parameter logistic fit or as logit B/B_0 *versus* log concentration using a linear fit. *NOTE: Cayman Chemical has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysis/elisa) to obtain a free copy of this convenient data analysis tool.*

Calculations

Preparation of the Data

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B_0 wells.
3. Subtract the NSB average from the B_0 average. This is the corrected B_0 or corrected maximum binding.
4. Calculate the B/B_0 (Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B_0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain $\%B/B_0$ for a logistic four-parameter fit, multiply these values by 100.)

NOTE: The TA values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; the corrected B_0 divided by the actual TA (100X measured absorbance) will give the %Bound. This value should closely approximate the %Bound that can be calculated from the ***Sample Data*** *(see page 27). Erratic absorbance values and a low (or no) %Bound could indicate the presence of organic solvents in the buffer or other technical problems (see page 31 for* ***Troubleshooting****).*

Plot the Standard Curve

Plot %B/B_0 for standards S1-S8 *versus* 11-dehydro TXB_2 concentration using linear (y) and log (x) axes and perform a 4-parameter logistic fit.

Alternative Plot - The data can also be lineraized using a logit transformation. The equation for this conversion is shown below. *NOTE: Do not use %B/B_0 in this calculation.*

$$\text{logit } (B/B_0) = \ln [B/B_0/(1 - B/B_0)]$$

Plot the data as logit (B/B_0) *versus* log concentrations and perform a linear regression fit.

Determine the Sample Concentration

Calculate the B/B_0 (or %B/B_0) value for each sample. Determine the concentration of each sample using the equation obtained from the standard curve plot. *NOTE: Remember to account for any concentration or dilution of the sample prior to the addition to the well.* Samples with %B/B_0 values greater than 80% or less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample indicates interference which could be eliminated by purification.

Performance Characteristics

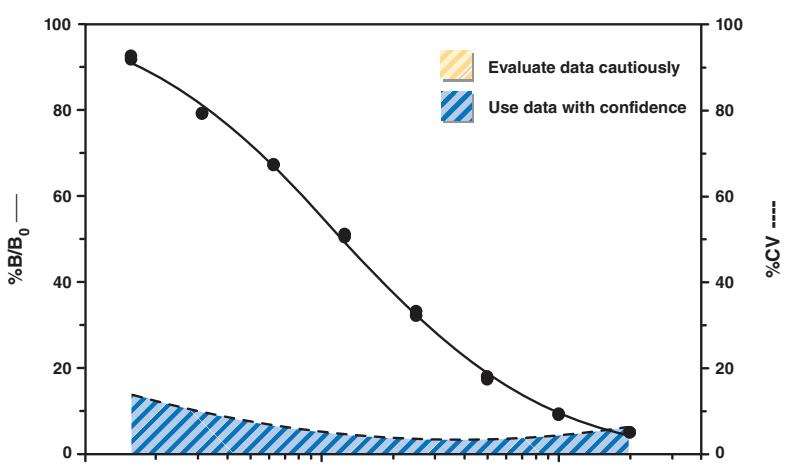
Sample Data

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples. Your results could differ substantially.

	Raw Data		Average	Corrected
Total Activity	0.595	0.538	0.567	
NSB	0.000	0.002	0.001	
B_0	1.176	1.267		
	1.191	1.182	1.204	1.203

Dose (pg/ml)	Raw Data		Corrected		%B/B_0	
2,000	0.061	0.062	0.060	0.061	5.0	5.0
1,000	0.114	0.110	0.113	0.109	9.3	9.1
500	0.210	0.218	0.209	0.217	17.4	18.0
250	0.388	0.401	0.387	0.400	32.1	33.2
125	0.608	0.616	0.607	0.615	50.5	51.1
62.5	0.812	0.809	0.811	0.808	67.4	67.2
31.3	0.954	0.955	0.953	0.954	79.2	79.3
15.6	1.104	1.116	1.103	1.115	91.7	92.6

Table 2. Typical results



Assay Range = 15.6-2,000 pg/ml
Sensitivity (defined as 80% B/B_0) = 34 pg/ml
Mid-point (defined as 50% B/B_0) = 80-160 pg/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted with Assay Buffer.

Figure 6. Typical standard curve

Precision:

The intra- and overnight intra-assay CVs have been determined at multiple points on the standard curve. The recommended two hour standard pre-incubation and optional overnight standard pre-incubation are summarized in the graph on page 28 and in the table below.

Dose (pg/ml)	%CV* Intra-assay variation	%CV* Intra-assay variation (with overnight/4°C Standard pre-incubation)
2,000	5.6	5.1
1,000	5.2	2.4
500	2.9	2.2
250	4.5	1.7
125	5.0	4.8
62.5	5.2	7.4
31.3	9.8	6.8
15.6	14.4	23.4

Table 3. Intra- and intra-assay variation
*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.

Cross Reactivity:

Compound	Cross Reactivity
11-dehydro Thromboxane B_2	100%
*11-dehydro-2,3-dinor Thromboxane B_2	330%
Prostaglandin D_2	0.12%
2,3-dinor Thromboxane B_2	0.10%
Thromboxane B_2	0.08%
Arachidonic Acid	<0.01%
Prostaglandin $F_{2\alpha}$	<0.01%

Table 4. Cross Reactivity of the 11-dehydro TXB_2 ELISA
*This assay measures both 11-dehydro TXB_2 and 11-dehydro-2,3-dinor TXB_2. The physiological revelance of these metabolites are similar.[13,14]

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique	A. Replace activated carbon filter or change source of UltraPure water
High NSB (>10% of B_0)	A. Poor washing B. Exposure of NSB wells to specific antibody	A. Rewash plate and redevelop
Very low B_0	A. Trace organic contaminants in the water source B. Plate requires additional development time C. Dilution error in preparing reagents	A. Replace activated carbon filter or change source of UltraPure water B. Return plate to shaker and re-read later
Low sensitivity (shift in dose response curve)	Standard is degraded	Replace standard
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present	Purify sample prior to analysis by ELISA[22]
Only Total Activity (TA) wells develop	Trace organic contaminants in the water source	Replace activated carbon filter or change source of UltraPure water

Additional Reading

Go to **www.caymanchem.com/519510/references** for a list of publications citing the use of Cayman's 11-dehydro Thromboxane B_2 ELISA Kit - Monoclonal.

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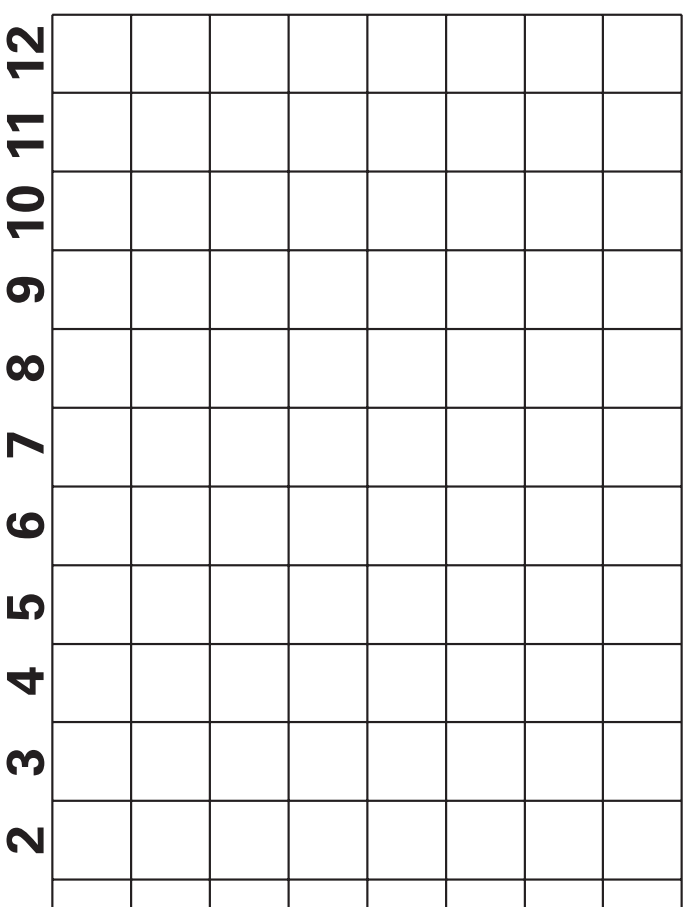

NOTES

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