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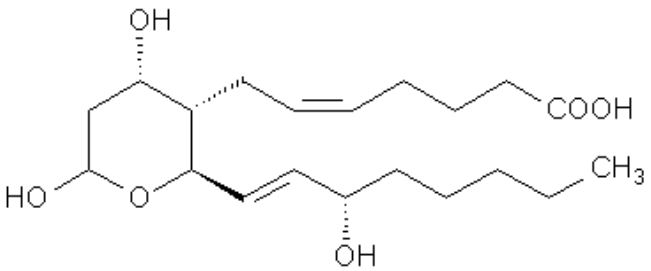
ProductInformation

Thromboxane B_2 EIA

Product Number **CS0190**
Storage Temperature –20 °C

Technical Bulletin

Product Description



11-dehydro TXB_2

Thromboxane B_2 EIA is a four-hour competitive enzyme immunoassay for the quantitative determination of Thromboxane B_2 (TXB_2) metabolite 11-dehydro TXB_2 concentrations in cell culture supernatants, saliva, urine, serum and plasma. TXB_2 present in the samples or standards competes with the fixed amount of TXB_2 conjugated to alkaline phosphatase for the limited number of binding sites on the anti-TXB_2 antibody. During the first incubation, the antigen-antibody complex binds to the anti-rabbit IgG antibody-coated multiwell plate. The excess conjugate and unbound sample are washed away and a substrate is added. During the second incubation the bound enzyme reacts with the substrate. The enzyme reaction is stopped and absorbance read at 405 nm. The intensity of the yellow color is inversely proportional to the concentration of TXB_2 in the standards or the samples. The concentration is calculated based on optical reading of standard dilution.

Thromboxane A_2 (TXA_2) is produced from arachidonic acid by many cells and causes irreversible platelet aggregation and vascular and bronchial smooth muscle contraction.[1] 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 plasma or urine is due to *ex vivo* platelet activation or intra-renal production. Normal concentrations of circulating TXB_2 are extremely low (1-2 pg/ml), and highly transient (t1/2 = 5-7 minutes). Therefore it is necessary to measure a metabolite that cannot be formed by platelets or by the kidney. Two metabolites 11-dehydro TXB_2, or 2,3-dinor TXB_2 are formed equally, although 11-dehydro TXB_2 has a longer circulating half-life (t1/2 = 45 minutes). Therefore, measurement of 11-dehydro TXB^2 in plasma or urine will give a time-integrated indication of TXA_2 production. Normal plasma levels of 11-dehydro TXB_2 (1-2 pg/mL) are below the detection limit of this assay (10 pg/mL), so plasma samples must be extracted prior to analysis. Because urine contains many crossreacting eicosanoid metabolites, it is advisable to extract urine also.

Reagents

- **Thromboxane B_2 (TXB_2) Standard, 1 vial, Product No T 8824** – 0.5 mL (100,000 pg/mL) in a buffer with preservative.
- **Anti-Rabbit IgG-coated 96 well plate, 1 plate, Product No. I 6283**
- **Assay Buffer, 30 mL, Product No. A 4228 –** contains sodium azide. Ready to use.
- **Anti-TXB_2, 6 mL, Product No. T 8574 –** from rabbit, contains sodium azide, yellow dye. Ready to use.
- **Thromboxane B_2 (TXB_2)-Alkaline Phosphatase Conjugate, 6 mL, Product No. T 8699 -** contains sodium azide, blue dye. Ready to use.
- **p-Nitrophenylphosphate (pNpp) Substrate, 20 mL, Product No. N 6911 -** Ready to use.
- **Wash Buffer Concentrate, 10X, 30 mL, Product No. W 3139 –** TRIS buffered saline with detergents and sodium azide.
- **Stop Solution, 5 mL, Product No. S 9443 –** a solution of trisodium phosphate. Ready to use.
- **Plate sealer, 1 each, Product No. P 1496**

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 405 nm, preferably with corrections between 570 and 590 nm.
- Horizontal orbital multiwell plates shaker capable of maintaining a speed of 500 +/- 50 rpm.
- Calibrated adjustable precision pipettes for volumes between 5 µL and 1,000 µL.
- If the sample requires extraction, cell extraction materials are needed (see recommended extraction procedure).
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard.
- Absorbent paper towels to blot the plate.
- Calibrated beakers and graduated cylinders in various sizes.
- Vortex mixer.
- Graph paper: linear, log-log, or semi-log, as desired.
- Thromboxane synthetase inhibitor, such as indomethacin (Prod. No. I 8280), or meclofenamic acid (Prod. No. M 4531)

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- Samples containing rabbit IgG may interfere with this assay.
- The assay is validated for cell culture samples, saliva, urine, plasma and serum.
- Samples may be assayed directly or after extraction.
- If samples are not assayed immediately, a Thromboxane synthetase inhibitor, such as indomethacin (Prod. No. I 8280), or meclofenamic acid (Prod. No. M4531) should be added to all samples at approximately 10 µg/mL before storage.
- Cell culture supernatants should be separated from the cells and frozen, if not analyzed shortly after collection. Avoid multiple freeze/thaw cycles.
- It may be possible to assay cell culture supernates (with or without serum supplement) containing high levels of 11-dehydro-TXB_2 without extraction. When running supernatants without extraction, dilute the kit standards in fresh culture media instead of Assay Buffer
- Use the same cell culture media for blanks, controls and standard dilutions.
- Samples, which normally have low levels of TXB_2, (below assay sensitivity) require extraction.
- Sera and plasma may require a 100-fold dilution in Assay Buffer.
- Urine and saliva samples require a 10-fold dilution in Assay Buffer.
- To ensure accurate results, always dilute the standards and blanks in the same diluent as the samples.

Materials Required for Extraction

- 2 M HCl
- Ethanol
- Hexane
- Ethyl acetate
- 200 mg C_{18} Reverse Phase Extraction Column
- High specific activity tritiated 11-dehydro TXB_2 (for determination of extraction efficiency).

Protocol for Sample Extraction

1. Acidify sample to pH 3.5 with 2N HCl. (~ 50 µL HCl per 1 mL of plasma).
2. Equilibrate at 4°C for 15 minutes.
3. Centrifuge samples in a microcentrifuge for 2 minutes to remove any precipitate.
4. Prepare the C_{18} reverse phase column by washing with 10 mL of ethanol followed by 10 mL of deionized water.
5. Apply the sample under a slight positive pressure to obtain a flow rate of about 0.5 mL/minute.
6. Wash the column with 10 mL of water, followed by 10 mL of 15% ethanol, and finally 10 mL hexane.
7. Elute the sample from the column by addition of 10 mL ethyl acetate.
8. If analysis is to be carried out immediately, evaporate samples under a stream of nitrogen.
9. Add 50 µL ethanol to the dried samples and reconstitute sample with at least 200 µL of Assay Buffer or cell culture media.
10. If analysis is to be delayed, store samples as the eluted ethyl acetate solutions at -70°C with until the time of assay.
11. Repeat steps 8,9.

Please refer to citations 8-9 for details of extraction protocols.

Reagent Preparation

TXB_2 Standard

1. Standards can be made either in glass or plastic tubes.
2. Pre-rinse pipette tips and change the tips before each dilution.
3. Equilibrate standard and all reagents to room temperature.
4. Prepare serial standard dilutions as follows:

Tube #	Assay Buffer or Cell Culture Media mL	Standard from tube #: mL	Final Standard Concentration pg/mL
0	Standard vial 100,000 pg/mL		
1	0.9 mL	0.1 mL (0)	10,000
2	0.75 mL	0.25 mL (1)	2,500
3	0.75 mL	0.25 mL (2)	625
4	0.75 mL	0.25 mL (3)	156.3
5	0.75 mL	0.25 mL (4)	39.1
6	0.75 mL	0.25 mL (5)	9.8

5. Diluted standards should be used within 60 minutes of preparation.

TXB_2 -Alkaline phosphatase conjugate

1. The activity of the TXB_2 conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions.
2. The activity of TXB_2 conjugate is affected by high concentrations of chelators, such as EDTA and EGTA. Samples that contain <10 mM EDTA or EGTA can be assayed without interference. Samples containing higher concentrations of chelators must be diluted prior to assay.
3. Equilibrate conjugate to room temperature before use.

Wash Buffer

1. Use only Wash Buffer provided in the kit.
2. Inorganic phosphate is a strong competitive inhibitor of alkaline phosphatase; **avoid the use of PBS-based wash buffers** and other sources of inorganic phosphate contamination.
3. Warm buffer to room temperature.
4. Dilute 10 mL wash concentrate with 90 mL deionized water.
5. Label as **Working Wash buffer**
6. Diluted buffer may be stored at room temperature for up to 3 months or until kit expiration date.

Storage/Stability

- The kit is shipped on dry ice.
- Store unopened kit at <–20 °C until use. Do not store past shelf life of the kit.
- After opening all components may be stored at 2-8 °C.

Refer to the Certificate of Analysis for shelf life of the kit. To obtain C of A go to www.sigma-aldrich.com

Procedure

Precautions

- Allow all reagents to equilibrate to room temperature (15-30 ºC) for at least 30 minutes before opening the kit.
- Use only the pre-coated 96 multiwell capture plate provided with the kit.
- Multiwell strips should be equilibrated to room temperature in the sealed foil bag.
- Remove desired number of strips, reseal the bag and refrigerate unused wells desiccated at 2 – 8°C to maintain plate integrity.
- When not in use all kit components should be stored at 2 to 8 °C**.**
- Do not use reagents after the shelf life of the kit.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Minimize contamination by endogenous alkaline phosphatase, present especially in the substrate solution. Avoid touching pipette tips and other items with bare hands.
- Alkaline Phosphatase is a temperature sensitive enzyme. Optical Density (OD) units may vary with temperature changes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.

- Pipette standards and samples to the bottom of the wells.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Read absorbances within 2 hours of assay completion.

Washing directions

1. The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
2. Incomplete washing will adversely affect the assay and render false results.
3. Avoid the use of phosphate-based buffers to prevent contamination with endogenous phosphate.
4. Washing may be performed using automated washer, manifold pipette or squirt bottle.
5. Wash cycle three times, blotting as dry as possible after the 3rd wash.
6. When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
7. It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

Refer to the diagram at the end of this bulletin to view the assay layout.

1st Incubation

a. Remove the appropriate number of strips and return the unused strips to the pouch. Reseal pouch and refrigerate.
b. Add 150 µL of Assay Buffer to the NSB wells
c. Add 100 µL of Assay Buffer to the zero standard (B_o) wells.
d. Add 100 µL of standards #1-6 to the appropriate wells.
e. Add 100 µL of samples to the appropriate wells
f. Add 50 µL of TXB_2-alkaline phosphatase conjugate to all wells (except the TA and SB).
g. Add 50 µL of Monoclonal Anti-TXB_2 to all wells (except NSB, TA and SB wells).
h. Cover with plate cover and incubate 2 hours at room temperature on orbital shaker set at 500 rpm.
i. All wells should be green, except NSB wells, which are blue.
j. Wash wells for a total of 3 times following washing instructions.
k. After the final wash, blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate Incubation

a. Add 5 µL of TXB_2 conjugate to the TA wells.
b. Add 200 µL of pNpp substrate to all wells. Cover.
c. Incubate 2 hours at 37 °C without shaking.

Stop Reaction

a. Add 50 µL of Stop Solution to each well.
b. Yellow color develops immediately and can be read in the multiwell plate reader at 405 nm with corrections at 570 or 590 nm.
c. Subtract the readings at 590 nm from the readings at 405 nm, to correct for optical imperfection of the plate.

Results

Average the duplicate readings for each standard and sample and subtract the average NSB optical density.

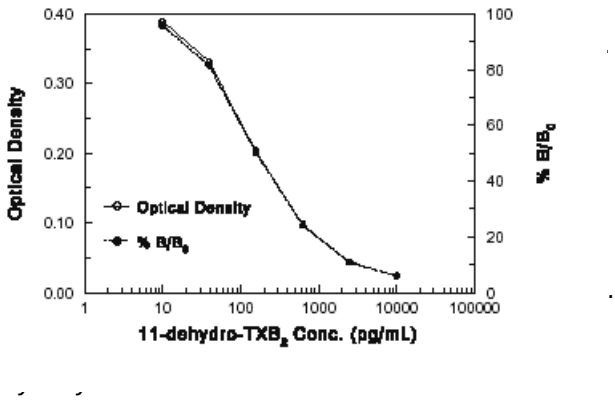
1. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit.
2. As an alternative, construct a standard curve by plotting the mean absorbance for each standard (nm) on a linear y-axis against the concentration (pg/mL) on a logarithmic x-axis and draw the best fit curve through the points on the graph.
3. % B/B_o can be calculated by dividing the corrected OD for each standard or sample by the corrected B_0 OD and multiplying by 100.
4. Calculate the concentration of TXB_2 corresponding to the mean absorbance or % B/B_o from the standard curve.
5. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Product Profile

Typical Results

Well ID	Mean OD (-Blank) 405 nm	Corrected Net OD nm	% Bound B/Bo	TXB_2 pg/mL
Blank OD	(0.152)			
TA	0.183	0.188		
NSB	0.077	0.000		
O (B_o)	0.483	0.603	100%	**0**
S1	0.101	0.024	5.9%	**10,000**
S2	0.121	0.044	10.8%	**2,500**
S3	0.175	0.098	24.1	**625**
S4	0.281	0.204	50.2	**156.3**
S5	0.408	0.331	81.5	**39.1**
S6	0.466	0.389	95.8%	**9.8**

Typical Standard Curve for TXB_2 EIA



Typical Quality Control Parameters

Substrate Blank (OD)	= 0.078
TA (TA x 10)	= 8.77
% NSB (NSB/TA x 100)	= 0.0%
% Bo (Bo/TA x 100)	= 4.6%
Quality of Fit	= 0.999

Performance Characteristics

Sensitivity

The sensitivity of the TXB_2 assay is typically less than 10.54 pg/mL. Sensitivity was determined by subtracting two standard deviations from the mean absorbance value of sixteen zero standard (B0) replicates and calculating the corresponding concentration.

Linearity

A sample containing 9000 pg/mL TXB_2 was diluted eight times 1:2 in the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual TXB_2 concentration versus measured TXB_2 concentration. The line obtained had a slope of 0.926 and a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of TXB_2 and running these samples 16 times in the same assay.
Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of TXB_2 in 8 different assays.

	TXB_2 pg/mL	Intra Assay %CV	Inter Assay %CV
Low	759	3.6	
Medium	1,283	4.0	
High	2,605	1.6	
Low	44		7.6
Medium	371		3.6
High	3,053		6.2

Recovery

The recovery of TXB_2 spiked into samples in various matrices was evaluated.

Sample	**% Recovery**	**Dilution**
Tissue Cultures	104.4	Undiluted
Human Saliva	98.3	1:10
Human Urine	102.2	1:10 to 1:100
Human Heparin Plasma	111.0	>1:100
Human Serum	113.7	>1:100

Cross Reactivities

The cross reactivities for a number of related compounds were determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer at concentrations from 39 to 500,000 pg/mL. These samples were measured in the TXB_2 assay and the TXB_2 concentration at 50% B/Bo calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	**Cross Reactivity**
TXB_2	100%
2,3-dinor TXB_2	7.1%
11-dehydro TXB_2	0,4%
6-keto $PGF_{1\alpha}$	0,23%
PGE_2	<0.01%
PGD_2	<0.01
$PGF_{1\alpha}$	<0.01
$PGF_{2\alpha}$	<0.01

References

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TXB_2 Assay Flow Chart (CS0190)

Well ID	**Blank**	**TA**	**NSB**	**B_0**	**Standards**	**Samples**
	A1, B1	**C1, D1**	**E1, F1**	**G1, H1**	**A2-C3**	**D3-H12**
Assay Buffer			150 µL	100 µL		
Standard and/or Sample					100 µL	100 µL
Conjugate			50 µL	50 µL	50 µL	50 µL
Anti-TXB_2 Antibody				50 µL	50 µL	50 µL

Incubate 2 hours @RT with shaking
Wash 3X, blot dry

Conjugate		5 µL				
pNpp Substrate	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL

Incubate 2 hours @ 37 ºC without shaking

Stop Solution	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL

Read at 405 nm