



Thromboxane B₂ ELISA kit

Catalog Number KA0294

96 assays

Version: 09

Intended for research use only

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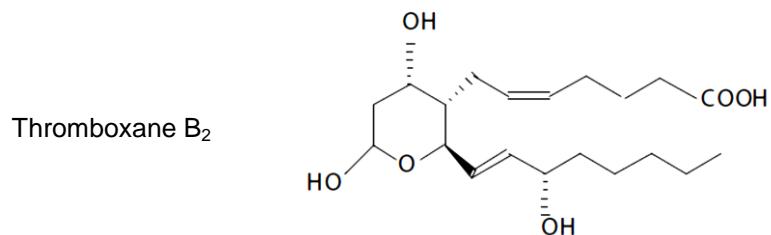
Introduction

Intended Use

The Thromboxane B₂ ELISA kit is a competitive immunoassay for the quantitative determination of Thromboxane B₂ in biological fluids. Please read the complete kit insert before performing this assay.

Background

Thromboxane A₂ (TXA₂) is involved in platelet aggregation, vasoconstriction and reproductive functions³⁻⁵. However TXA₂ has a half-life of only 37 seconds under physiological conditions⁵, and the production of TXA₂ in vivo is typically monitored by measurement of TXB₂ and 2,3-dinor TXB₂. Thromboxane B₂ (TXB₂) is produced by the non-enzymatic hydration of TXA₂, and has been shown to be stable^{6,7}. A number of pharmaceuticals alter and/or inhibit the synthesis of TXA₂⁸⁻¹⁰ and methods to determine TXA₂ in urine and blood typically involve gas chromatography/mass spectrometry¹¹, radioimmunoassay, or enzyme-linked immunosorbent assay.



Principle of the Assay

The kit for the quantitative measurement of Thromboxane B₂ uses a polyclonal antibody to TXB₂ to bind, in a competitive manner, the TXB₂ in the sample or an alkaline phosphatase molecule which has TXB₂ covalently attached to it. After a simultaneous incubation at room temperature the excess reagents are washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and the yellow color generated read on a microplate reader at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of TXB₂ in either standards or samples. The measured optical density for the samples is used to calculate the concentration of TXB₂ in the sample. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard¹ or Tijssen².

General Information

Materials Supplied

List of component

Component	Description	Amount
Goat anti-Rabbit IgG	A plate using break-apart strips coated with goat antibody specific to the Fc portion of rabbit IgG.	96 Wells
Microtiter Plate		
TXB ₂ ELISA Conjugate	A blue solution of alkaline phosphatase conjugated with TXB ₂ .	5 mL
TXB ₂ ELISA Antibody	A yellow solution of a rabbit polyclonal antibody to TXB ₂ .	5 mL
Assay Buffer	Tris buffered saline containing proteins and sodium azide as preservative.	27 mL
Wash Buffer Concentrate	Tris buffered saline containing detergents.	27 mL
Thromboxane B ₂ Standard	A solution of TXB ₂ in buffer containing 100,000 pg/mL.	0.5 mL
pNpp Substrate	A solution of p-nitrophenyl phosphate in buffer.	20 mL
Stop Solution	A solution of trisodium phosphate in water. Keep tightly capped. Caution: Caustic.	5 mL
Plate Sealer	-	1 slide

Storage Instruction

All components of this kit are stable at 4°C until the kit's expiration date.

Materials Required but Not Supplied

- ✓ Deionized or distilled water.
- ✓ Precision pipets for volumes between 5 µL and 1,000 µL.
- ✓ Repeater pipets for dispensing 50 µL and 200 µL.
- ✓ Disposable beaker for diluting Wash Buffer.
- ✓ Graduated cylinders.
- ✓ A microplate shaker.
- ✓ Adsorbent paper for blotting.
- ✓ Microplate reader capable of reading at 405 nm, preferably with correction at between 570 and 590 nm.

Precautions for Use

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

- Safety Warning & Precautions
- ✓ Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up.
- ✓ Stop solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
- ✓ The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.
- ✓ The Thromboxane B₂ Standard provided, is supplied in ethanolic buffer at a pH optimized to maintain TXB₂ integrity. Care should be taken handling this material because of the known and unknown effects of eicosanoids.
- Procedural Notes:
- ✓ Do not mix components from different kit lots or use reagents beyond the kit expiration date.
- ✓ Allow all reagents to warm to room temperature for at least 30 minutes before opening.
- ✓ Standards can be made up in either glass or plastic tubes.
- ✓ Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
- ✓ Pipet standards and samples to the bottom of the wells.
- ✓ Add the reagents to the side of the well to avoid contamination.
- ✓ This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.
- ✓ Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
- ✓ Prior to the addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in the assay results.

Assay Protocol

Reagent Preparation

- **TXB₂ Standard:**

Allow the 100,000 pg/mL TXB₂ standard solution to warm to room temperature. Label seven 12 x 75 mm glass tubes #1 through #7. Pipet 1000 µL of standard diluent (Assay Buffer or Tissue Culture Media) into tube #1. Pipet 666 µL of Standard diluent into tubes #2 through #7. Remove 100 µL of diluent from tube #1. Add 100 µL of the 100,000 pg/mL standard to tube #1. Vortex thoroughly. Add 333 µL of tube #1 to tube #2 and vortex thoroughly. Add 333 µL of tube #2 to tube #3 and vortex. Continue this for tubes #4 through #7. The concentration of TXB₂ in tubes #1 through #7 will be 10,000, 3,333, 1,111, 370, 123, 41.1, and 13.7 pg/mL respectively. See table below for dilution details. Diluted standards should be used within 60 minutes of preparation.

Std.	Assay Buffer Vol. (µL)	Vol. Added (µL)	TXB ₂ Conc. (pg/mL)
1	900	100, Stock	10,000
2	666	333, Std. 1	3,333
3	666	333, Std. 2	1,111
4	666	333, Std. 3	370
5	666	333, Std. 4	123
6	666	333, Std. 5	41.1
7	666	333, Std. 6	13.7

- **Wash Buffer:**

Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

Sample Preparation

- **Sample handing**

The TXB₂ enzyme-linked immunosorbent assay is compatible with TXB₂ samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer can be read directly from the standard curve. Please refer to the Sample Recovery recommendations for details of suggested dilutions. However, end user must verify that the recommended dilutions are appropriate for their samples. Samples containing rabbit IgG may interfere with the assay. Samples in the majority of tissue culture media, including those containing fetal bovine serum, can also be read in the assay, provided the standards have been diluted into the tissue culture media instead of Assay Buffer. There will be small change in binding associated with running the standards and samples in media. Users should only use standard curves generated in media or buffer to calculate concentrations of TXB₂ in the appropriate matrix. For tissue, urine and

plasma samples, prostaglandin synthetase inhibitors, such as, indomethacin or meclofenamic acid at concentrations up to 10 µg/mL should be added to either the tissue homogenate or urine and plasma samples. Urine samples may be used in the assay after dilution in Assay Buffer. Some samples may require extraction for accurate measurement. A suitable extraction procedure is outlined below:

- ✓ Materials Needed
 - 1. TXB₂ Standard to allow extraction efficiency to be accurately determined.
 - 2. 2 M hydrochloric acid, deionized water, ethanol, hexane and ethyl acetate.
 - 3. 200 mg C₁₈ Reverse Phase Extraction Columns.
- Procedure
 - 1. Acidify the plasma, urine or tissue homogenate by addition of 2 M HCl to pH of 3.5. Approximately 50 µL of HCl will be needed per mL of plasma. Allow to sit at 4°C for 15 minutes. Centrifuge samples in a microcentrifuge for 2 minutes to remove any precipitate.
 - 2. Prepare the C₁₈ reverse phase column by washing with 10 mL of >95% ethanol followed by 10 mL of deionized water.
 - 3. Apply the sample under a slight positive pressure to obtain a flow rate of about 0.5 mL/minute. Wash the column with 10 mL of water, followed by 10 mL of 15% ethanol, and finally 10 mL hexane. Elute the sample from the column by addition of 10 mL ethyl acetate.
 - 4. If analysis is to be carried out immediately, evaporate samples under a stream of nitrogen. Add at least 250 µL of Assay Buffer to the dried samples. Vortex well then allow to sit for five minutes. Repeat twice more. If analysis is to be delayed, store samples as the eluted ethyl acetate solutions at -80°C until the immunoassay is to be run. Evaporate the organic solvent under a stream of nitrogen prior to running assay and reconstitute as above.

Note: Please refer to references 12 and 13 for details of extraction protocols.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

1. Refer to the Plate Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4°C.
2. Pipet 100 µL of standard diluent (Assay Buffer or Tissue Culture Media) into the NSB and the Bo (0 pg/mL Standard) wells.
3. Pipet 100 µL of Standards #1 through #7 into the appropriate wells.
4. Pipet 100 µL of the Samples into the appropriate wells.
5. Pipet 50 µL of Assay Buffer into the NSB wells.
6. Pipet 50 µL of blue Conjugate into each well, except the Total Activity (TA) and Blank wells.
7. Pipet 50 µL of yellow Antibody Solution into each well, except the Blank, TA and NSB wells.

Note: Every well used should be Green in color except the NSB wells which should be Blue. The Blank and TA wells are empty at this point and have no color.

8. Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.
9. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 2 more times for a total of 3 washes.
10. After the final wash, empty or aspirate the wells and firmly tap the plate dry on a lint free paper towel to remove any remaining wash buffer.
11. Add 5 µL of the blue Conjugate to the TA wells.
12. Add 200 µL of the pNpp Substrate solution to every well. Incubate at room temperature for 45 minutes without shaking.
13. Add 50 µL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
14. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

- Assay protocol flow chart:

Well I.D.	Blank	TA	NSB	B0	Stds.	Samples
	A1, B1	C1, D1	E1, F1	G1, H1	A2-F3	G3-H12
Standard Diluent	---	---	100 µL	100 µL	---	---
Assay Buffer	---	---	50 µL	---	---	---
Std. and/or Sample	---	---	---	---	100 µL	100 µL
Conjugate	---	---	50 µL	50 µL	50 µL	50 µL
Antibody	---	---	---	50 µL	50 µL	50 µL
Incub. 2 hrs RT, shaking	→	→	→	→	→	→
Asp. & Wash 3 x 400 µL	→	→	→	→	→	→
Conjugate	---	5 µL	---	---	---	---
Substrate	200 µL					
Incub. 45 min. RT	→	→	→	→	→	→
Stop Solution	50 µL					

Data Analysis

Calculation of Results

Several options are available for the calculation of the concentration of TXB₂ in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If this sort of data reduction software is not readily available, the concentration of TXB₂ can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

$$\text{Average Net OD} = \text{Average OD} - \text{NSB OD}$$
2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

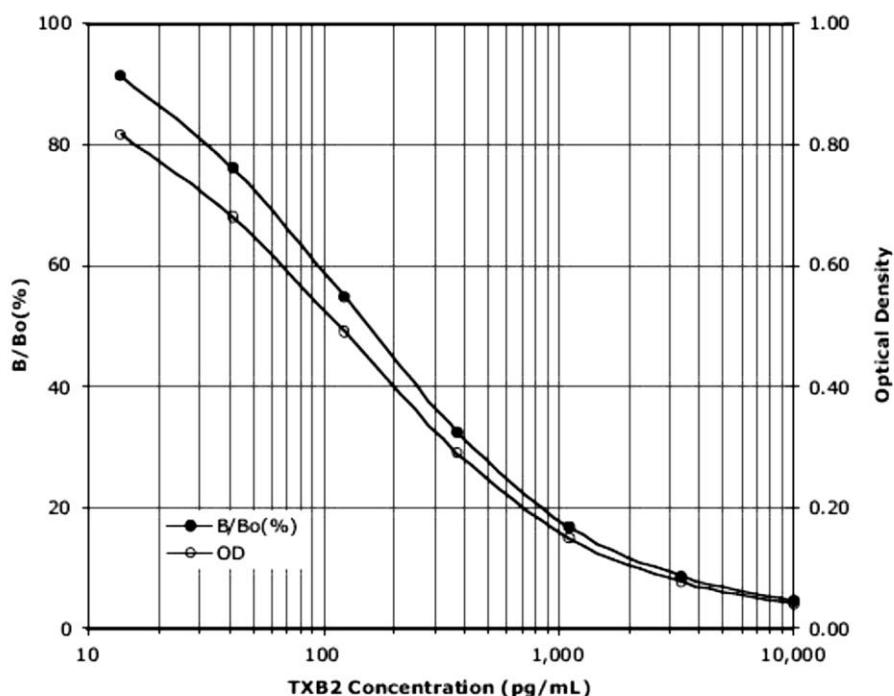
$$\text{Percent Bound} = (\text{Net OD} / \text{Net Bo OD}) \times 100$$
- Plot Percent Bound versus Concentration of TXB₂ for the standards. Approximate a straight line through the points. The concentration of TXB₂ in the unknowns can be determined by interpolation.
- Typical Results

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Mean OD (-Blank)	Average Net OD	Percent Bound	TXB ₂ (pg/mL)
Blank OD	(0.123)			
TA	0.693	0.570		
NSB	-0.003	0.000		
Bo	0.891	0.894	100%	0
S1	0.038	0.041	4.6%	10,000
S2	0.075	0.078	8.7%	3,333
S3	0.147	0.150	16.8%	1,111
S4	0.288	0.291	32.4%	370.3
S5	0.488	0.491	54.9%	123.4
S6	0.678	0.681	76.2%	41.15
S7	0.814	0.817	91.3%	13.7
Unknown 1	0.154	0.157		1,052
Unknown 2	0.410	0.413		184

- Typical Standard Curve

A typical standard curve is shown below. This curve must not be used to calculate TXB₂ concentrations; each user must run a standard curve for each plate used.



- Typical Quality Control Parameters

Total Activity Added	=	0.570 x 10 = 5.7
%NSB	=	-0.055%
%Bo/TA	=	15.7%
Quality of Fit	=	1.000 (Calculated from 4 parameter logistic curve fit)
20% Intercept	=	849 pg/mL
50% Intercept	=	153 pg/mL
80% Intercept	=	33 pg/mL

Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols¹⁴.

- **Sensitivity**

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run as Bo, and comparing to the average optical density for sixteen (16) wells run with Standard #7. The detection limit was determined as the concentration of TXB₂ measured at two (2) standard deviations from the zero along the standard curve.

Average Optical Density for the Bo = 0.613 ± 0.020 (2.68%)

Average Optical Density for Standard #7 = 0.561 ± 0.017 (2.46%)

Delta Optical Density (0-13.7 pg/mL) = $0.613 - 0.561 = 0.052$

2 SD's of the Zero Standard = $2 \times 0.020 = 0.040$

Sensitivity = $(0.040 / 0.052) \times 13.7 \text{ pg/mL} = 10.54 \text{ pg/mL}$

- **Linearity**

✓ A sample containing 3,239 pg/mL TXB₂ was diluted serially 1:2 seven times with kit Assay Buffer and measured in the assay. The data was plotted graphically as actual TXB₂ Concentration versus measured TXB₂ Concentration.

✓ The line obtained had a slope of 1.055 with a correlation coefficient of 0.999.

- **Precision**

✓ Intra-assay precision was determined by taking samples containing low, medium and high concentrations of TXB₂ and running these samples multiple times (n=24) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of TXB₂ in multiple assays (n=8).

✓ The precision numbers listed below represent the percent coefficient of variation for the concentrations of TXB₂ determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	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

- Cross Reactivities

The cross reactivities for a number of related eicosanoid compounds was determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer at concentrations from 500,000 to 13.7 pg/mL. These samples were then measured in the TXB₂ assay, and the measured TXB₂ 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 _{1α}	0.23%
PGD ₂	<0.01%
PGE ₂	<0.01%
PGF _{1α}	<0.01%
PGF _{2α}	<0.01%

- Sample Recoveries

- ✓ Please refer to Reagent and Sample Preparation sections.
- ✓ TXB₂ concentrations were measured in a variety of different samples, including tissue culture media, human saliva, serum, plasma and urine. For tissue culture media, saliva and urine samples TXB₂ was spiked into the undiluted samples, which were then diluted with the kit Assay Buffer. For human serum and plasma the endogenous levels of TXB₂ in the samples were used. The serum and plasma samples were diluted into the kit Assay Buffer and then assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Tissue Culture Media	104.7	None
Human Saliva	98.3	1:10
Human Urine	102.2	1:10-1:100
Human Heparin Plasma	111.0	>1:100
Human Serum	113.0	>1:100

* See Sample preparation instructions for details.

Resources

References

1. T. Chard,"An Introduction to Radioimmunoassay & Related Techniques 4th Ed.", (1990) Amsterdam:Elsevier.
2. P. Tijssen,"Practice & Theory of Enzyme Immunoassays", (1985) Amsterdam: Elsevier.
3. S. Moncada *et al.*, Pharm. Rev., (1978) 30: 293.
4. H. Sinzinger, *et al.*, Eicosanoids, (1990) 3: 59.
5. É.F. Smith III, Eicosanoids, (1989) 2: 199.
6. M.D. Mitchell, *et al.*, J. Endocrinol., (1978) 78: 435-441.
7. L. Viinikka & O. Ylikorkala, Prostaglandins, (1980) 20: 759-766.
8. E.K. Jackson, *et al.*, Arch. Pharmacol., (1988) 337: 183.
9. S. Conti, *et al.*, J. Pharm. Pharmacol., (1988) 40: 144.
10. K.D. Butler, *et al.*, Thrombosis Res., (1987) 45: 751.
11. G.A. FitzGerald, *et al.*, Adv. Prostaglandin & Thromboxane Res., (1985) 15: 87.
12. D.A. Weerasekera, *et al.*, Adv. Prostaglandin & Thromboxane Res., (1983) 11: 185-190.
13. H.R. Schroeder, *et al.*, Meth. Enzymol., (1978) 57: 424-445.
14. National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1, (1989) Villanova, PA: NCCLS.

Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Std 1	Std 5									
B	Blank	Std 1	Std 5									
C	TA	Std 2	Std 6									
D	TA	Std 2	Std 6									
E	NSB	Std 3	Std 7									
F	NSB	Std 3	Std 7									
G	B0	Std 4										
H	B0	Std 4										