



Kookaburra Thromboxane B₂ Enzyme Immunoassay Kit

Catalog No. 133-16463

96 Well Kit

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Description

Sapphire's Thromboxane B₂ (TXB₂) EIA kit is a competitive immunoassay for the quantitative determination of TXB₂ in biological samples. This assay is based on the competition between TXB₂ and a TXB₂-alkaline phosphatase tracer for a limited amount of TXB₂-specific antiserum. Because of the competition between TXB₂ in the sample and TXB₂ tracer for the TXB₂ antiserum, the signal obtained with the assay will be inversely proportional to the amount of TXB₂ in each sample. This equilibration is performed in the wells of a 96-well plate pre-coated with mouse anti-rabbit IgG, which binds all of the TXB₂ antiserum added to the well. After the incubation step, the plate is washed, and a solution of *para*-nitrophenyl phosphate (*p*NPP), a substrate for alkaline phosphatase, is added. The product of this enzymatic reaction has a distinct yellow color which absorbs at 412 nm, allowing quantification of TXB₂ in each sample.

Introduction

Thromboxane B₂ is a biologically-inactive, stable metabolite of TXA₂, a potent stimulator of vascular and bronchial smooth muscle contraction as well as irreversible platelet aggregation.¹⁻³ In response to a local stimulus, TXA₂ is produced from arachidonic acid by the combined action of cyclooxygenase (COX-1 or COX-2) and thromboxane synthase, exerting its effects within a short distance of its biosynthesis. TXA₂ is very unstable ($t_{1/2}$ ~30 seconds), being rapidly hydrolyzed non-enzymatically to TXB₂.⁴ It is difficult to accurately measure circulating levels of TXB₂ in whole-animals as plasma and urinary levels of TXB₂ are primarily due to *ex vivo* platelet activation and intra-renal production, respectively.⁵⁻⁷ TXB₂ can be metabolized by 2 different pathways – direct oxidation by 11-hydroxy thromboxane dehydrogenase leading to 11-dehydro TXB₂ or alternatively by β -oxidation to 2,3-dinor TXB₂.⁸

Precautions

1. Please read all instructions carefully before beginning this assay.
2. Some reagents contain azide, which may react with lead or copper plumbing. If disposing of reagents in the sanitary sewer, please flush with a large volume of water to prevent azide build-up.
3. This kit is for research use only. It is not intended for human or diagnostic use.

Materials Supplied

1. Thromboxane B ₂ Antiserum	1 vial	133-64633
2. Thromboxane B ₂ AP Tracer	1 vial	133-64632
3. Thromboxane B ₂ Standard	1 vial	133-64631
4. Mouse Anti-Rabbit IgG Coated Plate	1 plate	133-00008
5. Tris Buffer Concentrate	2 vials	133-00003
6. AP Wash Buffer Concentrate	1 vial	133-00004
7. DEA Buffer Concentrate	1 vial	133-00001
8. <i>p</i> NPP Tablets	5 tablets	133-00002
9. Plate Cover	1 cover	133-00005

Storage

This kit should be stored at -20°C and used before the expiration date printed on the box.

Materials Needed but Not Supplied

1. A source of Millipore water
2. Adjustable pipettors
3. Materials used for sample preparation
4. An orbital shaker
5. A plate reader capable of measuring absorbance between 405-420 nm

Sample Handling

[Note: Samples should be stored at -80°C if unable to be assayed immediately following collection.]

Materials needed for sample purification

1. TXB₂ standard to use in determination of recovery
2. 1.0 M HCl, methanol, Millipore water, hexane, and ethyl acetate
3. 6 ml SPE C-18 cartridges

Purification Procedure

While the following purification protocol works well for many samples, it may not be sufficient for the purification of TXB₂ from all samples.

1. Split samples into two equal parts and place each sample into clean test tubes.
2. Spike one of these sets of samples with TXB₂.
3. Perform the following steps on all samples:
 - a. Acidify the sample to pH <4.0 by the addition of 1.0 M HCl.
 - b. Prepare a C-18 SPE cartridge by conditioning it first with 5 ml of methanol followed by 5 ml Millipore water.
 - c. Apply the sample and allow to flow through the column.
 - d. Rinse the column with 5 ml Millipore water, followed by 5 ml hexane. Allow the column to become dry following the hexane wash.
 - e. Elute the TXB₂ with 5 ml ethyl acetate containing 1% methanol. If unable to run the assay at this time, store the samples in the ethyl acetate/methanol at -80°C.
 - f. Dry the sample under a stream of nitrogen. Reconstitute the sample in Tris Buffer and assay both the unspiked and spiked samples in the EIA.

Reagent Preparation

All diluted buffers should be stored at 4°C. When stored in this manner, they will be stable for approximately two months.

1. Tris Buffer

Dilute the contents of one vial of Tris Buffer Concentrate with 90 ml of Millipore water. It is common for the concentrated buffer to contain crystalline salts after thawing. It is important to rinse the vial to obtain any salts that may have precipitated.

2. Wash Buffer

Dilute the 5 ml vial of Wash Buffer to a final volume of 750 ml with Millipore water.

3. DEA Buffer

Dilute the 2.5 ml vial of DEA Buffer Concentrate to a final volume of 25 ml with Millipore water.

4. Thromboxane B₂ Standard

[Note: If assaying culture medium samples that have not been diluted with Tris Buffer, the culture medium rather than Tris Buffer should be used for dilution of the standard curve.]

Equilibrate a pipet tip in ethanol, by repeatedly filling and expelling. Use the equilibrated pipet tip to transfer 100 µl of the TXB₂ standard into a clean test tube. Dilute with 900 µl Millipore water. The concentration of this standard is 20 ng/ml. Label eight glass test tubes #1 - #8. Pipet 900 µl of Tris Buffer into tube #1, and 500 µl into tubes #2 - #8. Transfer 100 µl of the 20 ng/ml standard into tube #1 and vortex to mix. Transfer 500 µl from tube #1 to tube #2. Vortex to mix. Transfer 500 µl from tube #2 to tube #3. Vortex to mix. Continue this process for standard tubes #4 - #8. These diluted standards should be used within twenty-four hours.

5. Thromboxane B₂ Alkaline Phosphatase Tracer

Reconstitute the TXB₂ Alkaline Phosphatase Tracer with 6 ml of Tris buffer. Vortex to mix.

Store this reconstituted tracer at 4°C and use within four weeks.

6. Thromboxane B₂ Antiserum

Reconstitute the TXB₂ Antiserum with 6 ml of Tris Buffer. Vortex to mix.

Store this reconstituted antiserum at 4°C and use within four weeks.

Assay Procedure

[*Note: All reagents should be allowed to warm to room temperature before use.*]

1. Tris Buffer

Pipet 150 µl Tris Buffer into non-specific binding (NSB) wells, and 100 µl Tris Buffer into zero standard (B₀) wells. If tissue culture medium was used to dilute the standard curve, substitute 100 µl of this same medium for the Tris Buffer in the NSB and B₀ wells.

2. Thromboxane B₂ Standard

Pipet 100 µl of standards into the appropriate wells.

3. Samples

Pipet 100 µl of samples into the appropriate wells. Each sample should be assayed in duplicate or triplicate.

4. Thromboxane B₂ Alkaline Phosphatase Tracer

Pipet 50 µl of tracer into each well except the blank wells and total activity (TA) wells.

5. Thromboxane B₂ Antiserum

Pipet 50 µl of antiserum into each well except the blank wells, TA wells and NSB wells.

Well	Tris Buffer	Std/Sample	Tracer	Antiserum
Blank				
TA			5 µl (at development)	
NSB	150 µl		50 µl	
B ₀	100 µl		50 µl	50 µl
Std/Sample		100 µl	50 µl	50 µl

Pipetting Summary

6. Incubate the plate

Cover each plate with a plate cover and incubate for two hours at room temperature on an orbital shaker.

7. Prepare the pNPP Solution

Dissolve 5 pNPP tablets in 25 ml DEA buffer (25 ml is sufficient to develop 100 wells). *Note: Reconstituted pNPP is not stable, so we recommend that you make only the amount that you need at any one time.*

8. Wash the plate

Empty the wells and rinse five times with wash buffer. After the final wash, firmly tap the inverted plate on a paper towel to remove any recalcitrant drops of buffer.

9. Develop the plate

Add 200 µl pNPP solution to each well including blank and TA wells. Add 5 µl of tracer to the TA wells. Cover the plate and allow to develop in the dark on an orbital shaker. This assay typically develops in approximately 60-90 minutes.

10. Read the plate

Wipe the bottom of the plate with a paper towel to remove any finger prints, smudges or dirt which may interfere with obtaining an accurate reading of absorbance. Remove the plate cover, and read the plate at a wavelength of between 405 and 420 nm.

Data Analysis

Most plate readers come with data reduction software that plots data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as %B/B₀ versus log concentration of standard using either a 4-parameter logistic or a log-logit curve fit.

1. Prepare the Data

The following procedure is recommended to prepare the data prior to graphing (subtract the absorbance of the blank wells from all wells on the plate, if not already done).

- a. Average the absorbance readings from the NSB wells.
- b. Average the absorbance readings from the B₀ wells.
- c. Subtract the average NSB from the average B₀. This is the corrected B₀.

$$\text{Corrected B}_0 = \text{Average B}_0 - \text{Average NSB}$$

- d. Calculate the %B/B₀ for each standard and sample. To do this, subtract the average NSB absorbance from the absorbance and divide by the corrected B₀ (from step c). Multiply by 100 to obtain %B/B₀. Repeat for all wells.

$$\%B/B_0 = \left[\frac{\text{Absorbance} - \text{Average NSB}}{\text{Corrected B}_0} \right] \times 100$$

2. Plot the Standard Curve

Plot %B/B₀ for all standards versus TXB₂ concentration using log (x) and linear (y) axes, and fit the data to a four parameter logistic equation. Alternatively, the data can be linearized using a logit transformation. [*Note: Do not use %B/B₀ in this calculation.*]

$$\text{Logit (B/B}_0) = \ln [B/B_0/(1-B/B_0)]$$

Plot the data as logit(B/B₀) versus log concentration of standard and perform a linear regression fit.

3. Determine the Concentration of your Samples

Calculate the %B/B₀ for each sample. Determine the concentration of each sample using the equations obtained from the analysis of the standard curve. Remember to account for any dilutions made to the sample prior to addition to the well. %B/B₀ values of greater than 80% or less than 20% should be re-assayed as they generally fall outside of the linear range of the standard curve.

4. Correct for Recovery (if purification was performed)

Divide the concentration determined in step #3 by the recovery factor. Correct for any volume changes of the sample which may have occurred during purification.

Recovery Factor =

$$\frac{\text{EIA value of spiked sample (pg/ml)} - \text{EIA value of unspiked sample (pg/ml)}}{\text{Concentration of spike (pg/ml)}}$$

Assay Performance Characteristics

1. Precision

Intra-assay precision was determined by measuring samples containing low, medium, and high concentrations of TXB₂ multiple times in the same assay (eight samples per plate on a total of five plates). Inter-assay precision was determined by measuring low, medium, and high concentrations of the samples eight times using different reagents.

	TXB ₂ (pg/ml)	Intra-assay %CV	Inter-assay %CV
High	250	9.3	4.9
Medium	125	12.2	5.2
Low	62.5	11.3	5.8

2. Specificity

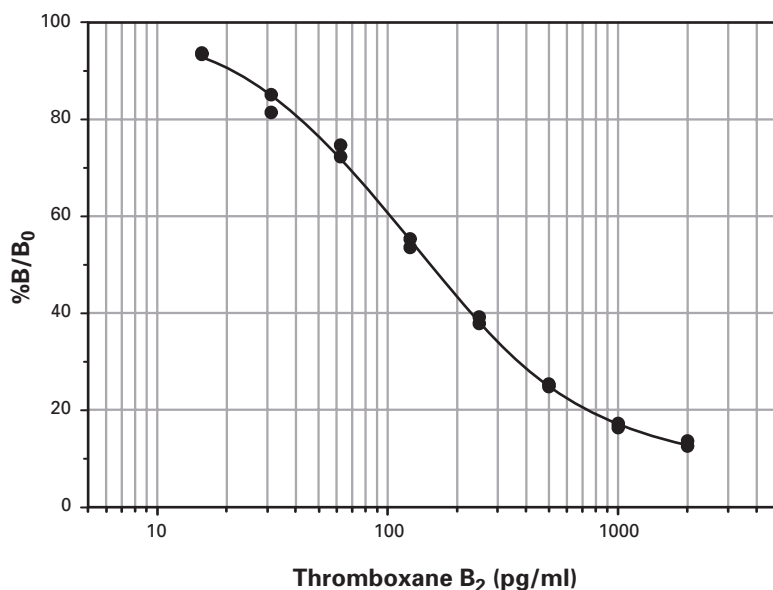
Analyte	Cross-Reactivity	Analyte	Cross-Reactivity
Thromboxane B ₂	100%	Prostaglandin E ₂	0.09%
Thromboxane B ₃	200%	6-keto Prostaglandin F _{1α}	0.08%
2,3-dinor Thromboxane B ₂	9.9%	Leukotriene B ₄	<0.01%
11-dehydro Thromboxane B ₃	0.62%	2,3-dinor-6-keto Prostaglandin F _{1α}	<0.01%
Prostaglandin D ₂	0.53%	13,14-dihydro-15-keto Prostaglandin F _{1α}	<0.01%
11-dehydro Thromboxane B ₂	0.42%	Tetranor PGEM	<0.01%
Prostaglandin F _{2α}	0.25%	Tetranor PGFM	<0.01%
Prostaglandin F _{1α}	0.11%		

3. Typical Results

The standard curve shown here is an example of data typically produced by this kit. Your results will vary from these, and it is therefore important that you run a standard curve each time you use the kit.

	Raw Data		Average	Corrected
Total Activity	3.670	3.567	3.619	
NSB	0	0	0	
B ₀	0.582	0.586	0.577	0.577
	0.576	0.564		

Concentration Std (pg/ml)	Raw Data		Corrected		%B/B ₀	
2,000	0.072	0.078	0.072	0.078	12.5	13.5
1,000	0.094	0.099	0.094	0.099	16.3	17.2
500	0.142	0.146	0.142	0.146	24.8	25.3
250	0.218	0.226	0.218	0.226	37.8	39.2
125	0.309	0.319	0.309	0.319	53.6	55.3
62.5	0.431	0.417	0.431	0.417	74.7	72.3
31.3	0.491	0.470	0.491	0.470	85.1	81.5
15.6	0.539	0.541	0.539	0.541	93.4	93.8



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