



ELISA Kit
Catalog # KHL1781

Human
Thromboxane B₂

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INTRODUCTION

Thromboxane B₂ (TXB₂) 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 α -oxidation to 2,3-dinor TXB₂.⁸

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READ ENTIRE PROTOCOL BEFORE USE

PRINCIPLE OF THE METHOD

The Invitrogen Human Thromboxane B₂ (TXB₂) ELISA 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 microtiter 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.

REAGENTS PROVIDED

Note: This kit should be stored at -20°C and used before the expiration date.

<i>Reagent</i>	<i>96 Test Kit</i>
<i>Thromboxane B₂ Antiserum</i> , lyophilized. Contains 0.05% sodium azide.	1 vial
<i>Thromboxane B₂ AP Tracer</i> , lyophilized. Contains 0.05% sodium azide.	1 vial
<i>Thromboxane B₂ Standard</i> in ethanol; 0.5 mL per vial.	1 vial
<i>Mouse Anti-rabbit IgG Coated Plate</i> , 96 wells per plate.	1 plate
<i>Tris Buffer Concentrate</i> . Contains 0.5% sodium azide; 10 mL per vial.	2 vials
<i>Wash Buffer Concentrate</i> ; 5 mL per vial.	1 vial
<i>DEA Buffer Concentrate</i> ; 2.5 mL per vial.	1 vial
<i>pNPP Tablets</i> .	5 tablets
<i>Plate Cover</i> , adhesive strips.	1

Disposal Note: This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

SUPPLIES REQUIRED BUT NOT PROVIDED

1. Ultrapure (18 M Ω) distilled or deionized water.
2. Calibrated adjustable precision pipettes, preferably with disposable plastic tips. (A manifold-channel pipette is desirable for large assays.)
3. An orbital shaker.
4. A plate reader capable of measuring between 405-420 nm.
5. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
6. Glass tubes for diluting and aliquoting standard.
7. Absorbent paper towels.
8. Calibrated beakers and graduated cylinders in various sizes.
9. 1.0 M HCl.
10. Methanol.
11. Hexane.
12. Ethyl acetate.
13. SPE C-18 cartridges.

PROCEDURAL NOTES/LAB QUALITY CONTROL

1. **Microtiter plates should be allowed to come to room temperature before opening the foil bags.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.
2. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
3. If particulate matter is present, centrifuge or filter prior to analysis.
4. All standards, controls and samples should be run in duplicate.

5. Samples that are greater than the highest standard point should be diluted and retested.
6. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
7. Cover or cap all reagents when not in use.
8. **Do not mix or interchange different reagent lots from various kit lots.**
9. Do not use reagents after the kit expiration date.
10. Read absorbances within 2 hours of assay completion.
11. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
12. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. *Never* insert absorbent paper directly into the wells.

SAFETY

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

DIRECTIONS FOR WASHING

Incomplete washing will adversely affect the test outcome. All washing must be performed with *Wash Buffer* provided.

Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into the bottom of each well. Take care not to scratch the inside of the well.

After aspiration, fill the wells with at least 0.4 mL of diluted *Wash Buffer*. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under **ASSAY METHOD**. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

Alternatively, the *Wash Buffer* may be put into a squirt bottle. If a squirt bottle is used, flood the plate with *Wash Buffer*, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

If using an automated washer, the operating instructions for washing equipment should be carefully followed. If your automated washer allows, 30 second soak cycles should be programmed into the wash cycle.

SAMPLE PREPARATION

Purification Procedure:

While the following purification protocol works well for many samples, it is important to be aware that 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 SPE C-18 cartridge by conditioning it first with 5 mL of methanol followed by 5 mL distilled or deionized water.
 - c. Apply the sample and allow to flow through the column.
 - d. Rinse the column with 5 mL distilled or deionized 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 ethyl acetate/methanol at -80°C.
 - f. Dry the sample under a stream of nitrogen. Reconstitute the sample in 1x Tris Buffer. Assay both unspiked and spiked samples with the ELISA.

REAGENT PREPARATION AND STORAGE

- A. All diluted buffers should be stored at 2 to 8°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 distilled or deionized 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 Concentrate* to a final volume of 750 mL with distilled or deionized water.
- 3. DEA Buffer.**
Dilute the 2.5 mL vial of *DEA Buffer Concentrate* to a final volume of 25 mL with distilled or deionized 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 pipette tip in ethanol, by repeatedly filling and expelling. Use the equilibrated pipette tip to transfer 100 μ L of the *TXB₂ Standard* into a clean test tube. Dilute with 900 μ L distilled or deionized water. The concentration of this stock standard is 20 ng/mL. Label eight glass test tubes #1 through #8. Pipette 900 μ L of 1x Tris Buffer into tube #1, and 500 μ L into tubes #2 through #8. Transfer 100 μ L of the 20 ng/mL standard into tube #1 and vortex to mix. Transfer 500 μ L from tube #1 to tube #2. Vortex to mix. Transfer 500 μ L from tube #2 to tube #3. Vortex to mix. Continue this process for standard tubes #4 through #8. These diluted standards should be used within twenty-four hours.

B. Dilution of Thromboxane B₂ Standard

Standard:	Add:	Into:
2 ng/mL	100 µL of 20 ng/mL stock std. prepared in Step 4.	900 µL 1x Tris Buffer
1 ng/mL	500 µL of the 2 ng/mL std.	500 µL 1x Tris Buffer
0.5 ng/mL	500 µL of the 1 ng/mL std.	500 µL 1x Tris Buffer
0.25 ng/mL	500 µL of the 0.5 ng/mL std.	500 µL 1x Tris Buffer
0.125 ng/mL	500 µL of the 0.25 ng/mL std.	500 µL 1x Tris Buffer
0.06 ng/mL	500 µL of the 0.125 ng/mL std.	500 µL 1x Tris Buffer
0.03 ng/mL	500 µL of the 0.06 ng/mL std.	500 µL 1x Tris Buffer
0.016 ng/mL	500 µL of the 0.03 ng/mL std.	500 µL 1x Tris Buffer

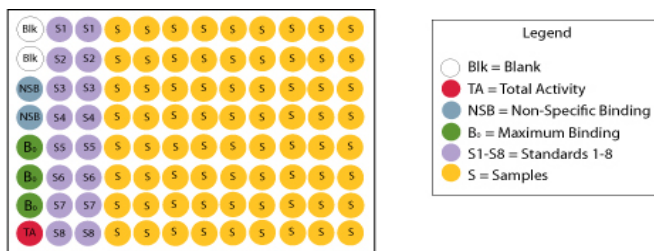
Diluted standards may be stored at 2 to 8°C for up to 24 hours.

5. **Thromboxane B₂ Alkaline Phosphatase Tracer.**
Reconstitute the *TXB₂ Alkaline Phosphatase Tracer* with 6 mL of 1x Tris buffer. Vortex to mix. Store this reconstituted tracer at 2 to 8°C and use within four weeks.
6. **Thromboxane B₂ Antiserum.**
Reconstitute the *TXB₂ Antiserum* with 6 mL of 1x Tris Buffer. Vortex to mix. Store this reconstituted antiserum at 2 to 8°C and use within four weeks.

ASSAY METHOD: PROCEDURE

(Note: All reagents should be allowed to warm to room temperature before use.)

Figure 1: Suggested Plate Plan.



1. Pipette 150 µL 1x Tris Buffer into non-specific binding (NSB) wells, and 100 µL 1x Tris Buffer into zero standard (B₀) wells. If tissue culture medium was used to dilute the standard, substitute 100 µL of this same medium for the Tris Buffer in the NSB and B₀ wells.

2. Pipette 100 μL of *Thromboxane B₂ Standard* into the appropriate wells.
3. Pipette 100 μL of samples into the appropriate wells. Each sample should be assayed in duplicate or triplicate.
4. Pipette 50 μL of *Thromboxane B₂ Alkaline Phosphatase Tracer* into each well except the blank wells and total activity (TA) wells.
5. Pipette 50 μL of *Thromboxane B₂ Antiserum* into each well except the blank wells, TA wells and NSB wells

Note: The table below specifies reagents required for designated wells. Reading across the table, confirm you added the correct reagent(s) to each well.

Well	Tris Buffer	Std/Sample	Tracer	Antibody
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

DEFINITIONS OF KEY TERMS

Blank - No sample; to measure background absorbance.

TA (Total Activity) - total activity of the AP-linked tracer.

NSB (Non-Specific Binding) - non-immunological binding of the tracer to the well.

B₀ (Maximum Binding) - maximum amount of the tracer that the antiserum can bind in the absence of the analyte.

6. Cover each plate with a plate cover and incubate for two hours at room temperature on an orbital shaker.
7. Dissolve 5 pNPP tablets in 25 mL 1x 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. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 5 times. See **DIRECTIONS FOR WASHING**.
9. Add 200 μ L pNPP solution to each well including blank and TA wells. Add 5 μ L 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 to 90 minutes.
10. 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 absorbance reading. 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:

- a. Subtract the absorbance of the blank wells from all wells on the plate, if not already done.
- b. Average the absorbance readings from the NSB wells.
- c. Average the absorbance readings from the B₀ wells.
- d. Subtract the average NSB from the average B₀. This is the corrected B₀.

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

- e. Calculate the %B/B₀ for each standard and sample. To do this, subtract the average NSB absorbance from the standard and sample absorbances and divide by the corrected B₀ (from step d). 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.*]

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

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 dilution 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).

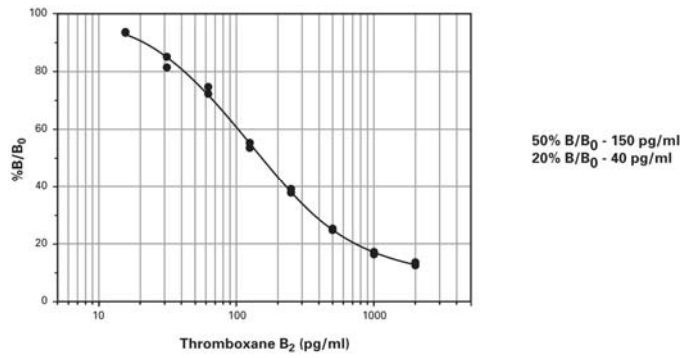
Divide the concentration determined in step #3 by the recovery factor. Correct for any volume changes of the sample which may have occurred during purification.

$$\text{Recovery Factor} = \frac{\text{ELISA value of spiked (pg/mL)} - \text{ELISA value of unspiked sample (pg/mL)}}{\text{Concentration of spike (pg/mL)}}$$

TYPICAL DATA

The standard curve is an example of data typically produced by this kit (Figure 2). Your results will vary from these, and it is therefore important that you run a standard curve each time you use the kit.

Figure 2: Standard curve for Thromboxane B₂



LIMITATIONS OF THE PROCEDURE

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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 in eight separate assays.

	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

Buffered solutions of a panel of substances were assayed. The following substances were tested and their corresponding cross reactivity percentages are noted.














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%		

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Explanation of symbols

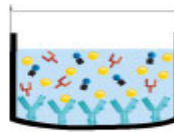
Symbol	Description	Symbol	Description
	Catalogue Number		Batch code
	Research Use Only		<i>In vitro</i> diagnostic medical device
	Use by		Temperature limitation
	Manufacturer		European Community authorised representative
	Without, does not contain		With, contains
	Protect from light		Consult accompanying documents
	Directs the user to consult instructions for use (IFU), accompanying the product.		

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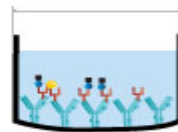
NOTES

Thromboxane B₂ Assay Summary

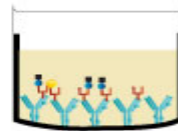
Incubate with 50 μ l tracer, 50 μ l antiserum, and either 50 μ l standard or unknown sample, for 2 hours at RT.



Wash to remove all unbound reagents.



Add pNPP solution and incubate for 60-90 minutes.



Read Plate between 405-420 nm
Total time: 3 hours

