ab133022 – Thromboxane B_2 ELISA Kit

Instructions for Use

For quantitative detection of Thromboxane B_2 (TXB_2) in biological fluids.

This product is for research use only and is not intended for diagnostic use.

Version 10b Last Updated 1 October 2021

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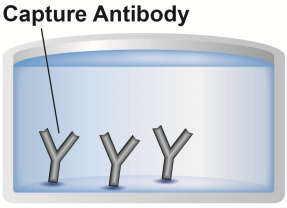
1. BACKGROUND

Abcam's Thromboxane B_2 (TXB_2) *in vitro* competitive ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of Thromboxane B_2 in biological fluids.

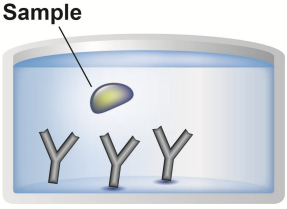
A goat anti-rabbit IgG antibody has been precoated onto 96-well plates. Standards or test samples are added to the wells, along with an alkaline phosphatase (AP) conjugated- TXB_2 antigen and a rabbit polyclonal antibody specific to TXB_2. After incubation the excess reagents are washed away and pNpp substrate is added. After a short incubation the enzyme reaction is stopped and a yellow color is generated. The intensity of the yellow coloration is inversely proportional to the amount of TXB_2 captured in the plate.

Thromboxane A_2 (TXA_2) is involved in platelet aggregation, vasoconstriction and reproductive functions. However TXA_2 has a half life of only 37 seconds under physiological conditions, and the production of TXA_2 in vivo is typically monitored by measurement of TXB_2 and 2,3-dinor TXB_2. Thromboxane B_2 (TXB_2) is produced by the non-enzymatic hydration of TXA_2, and has been shown to be stable. A number of pharmaceuticals alter and/or inhibit the synthesis of TXA_2 and methods to determine TXA_2 in urine and blood typically involve gas chromatography/mass spectrometry, radioimmunoassay, or enzyme immunoassay.

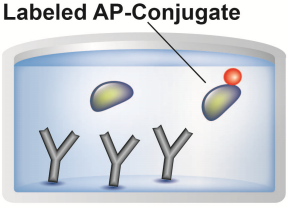
2. ASSAY SUMMARY



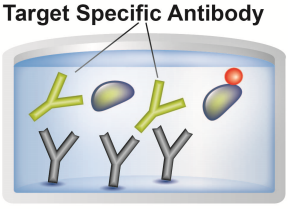
Prepare all reagents and samples as instructed.



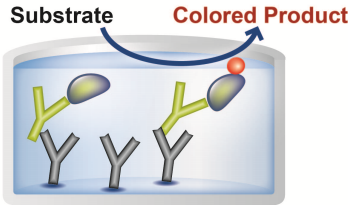
Add standards and samples to appropriate wells.



Add prepared labeled AP-conjugate to appropriate wells.



Add TXB_2 antibody to appropriate wells. Incubate at room temperature.



Add pNpp substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- 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
- We test the performance of this kit with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results
- The TXB_2 Standard provided, is supplied in ethanolic buffer at a pH optimized to maintain TXB_2 integrity. Care should be taken handling this material because of the known and unknown effects of eicosanoids.

4. STORAGE AND STABILITY

Store kit at +4°C immediately upon receipt.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Goat anti-rabbit IgG Microplate (12 x 8 wells)	96 Wells	+4ºC
TXB_2 Alkaline Phosphatase Conjugate	5 mL	+4ºC
Rabbit polyclonal TXB_2 Antibody	5 mL	+4ºC
TXB_2 Standard	500 µL	+4ºC
Assay Buffer	27 mL	+4ºC
20X Wash Buffer Concentrate	27 mL	+4ºC
pNpp Substrate	20 mL	+4ºC
Stop Solution	5 mL	+4ºC
Plate Sealer	1 Unit	+4ºC

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Standard microplate reader - capable of reading at 405 nm, preferably with correction between 570 and 590 nm.
- Automated plate washer (optional)
- Adjustable pipettes and pipette tips. Multichannel pipettes are recommended when large sample sets are being analyzed
- Eppendorf tubes
- Microplate Shaker
- Absorbent paper for blotting
- 200 mg C18 Reverse Phase Extraction Columns (only required for extraction of samples containing low levels of TXB_2)
- 2M hydrochloric acid (only required for extraction of samples containing low levels of TXB_2)
- Deionized water
- Ethanol
- Hexane
- Ethyl acetate

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted

8. TECHNICAL HINTS

- Standards can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent, use fresh pipette tips for each sample, standard and reagent
- Pipette 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 addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18 - 25°C) prior to use.

9.1 1X Wash Buffer

Prepare the 1X Wash Buffer by diluting 5 mL of the 20X Wash Buffer Concentrate in 95 mL of deionized water. Mix thoroughly and gently. 1X Wash buffer can be stored at room temperature for three months, or until the expiration date of the kit, whichever is earlier.

10. STANDARD PREPARATIONS

Prepare serially diluted standards in glass or plastic tubes immediately prior to use. Always prepare a fresh set of standards for every use. Diluted standards should be used within 60 minutes of preparation.

10.1 Allow the 100,000 pg/mL TXB_2 **Stock Standard** solution to equilibrate to room temperature. The standard solution should be aliquoted and stored at -20°. Avoid repeated freeze-thaw cycles. Dilute standards with the appropriate diluent for the samples being analyzed (either assay buffer or tissue culture media).

10.2 Label seven tubes #1 – #7.

10.3 Add 900 µL of appropriate diluent (Assay Buffer or Tissue Culture Media) to tube #1.

10.4 Add 666 µL appropriate diluent to tubes 2 through 7.

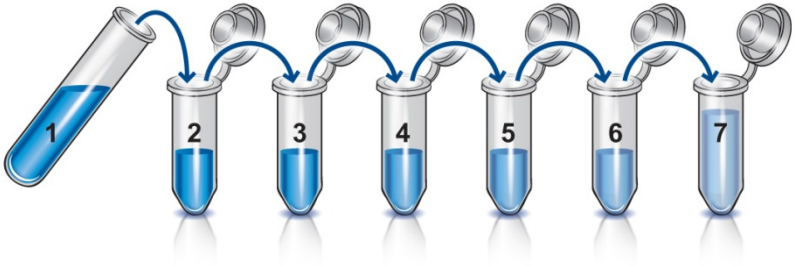
10.5 Prepare a 10,000 pg/mL **Standard 1** by adding 100 µL of the 100,000 pg/mL Stock Standard to tube #1. Vortex thoroughly.

10.6 Prepare **Standard 2** by transferring 333 µL from tube #1 to tube #2. Mix thoroughly and gently.

10.7 Prepare **Standard 3** by transferring 333 µL from tube #2 to tube 3. Mix thoroughly and gently.

10.8 Using the table below as a guide, repeat for tubes #4 through #7.

Standard #	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	Stock	100	900	100,000	10,000
2	Standard 1	333	666	10,000	3,333
3	Standard 2	333	666	3,333	1,111
4	Standard 3	333	666	1,111	370
5	Standard 4	333	666	370	123
6	Standard 5	333	666	123	41.1
7	Standard 6	333	666	41.1	13.7



11. SAMPLE COLLECTION AND STORAGE

- The TXB_2 enzyme immunoassay is compatible with TXB_2 samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer can be read directly from the standard curve. 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 TXB_2 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:

11.1 Acidify the plasma, urine or tissue homogenate by addition of 2M 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

11.2 Prepare the C18 reverse phase column by washing with 10 mL of ethanol followed by 10 mL of deionized water.

11.3 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.

11.4 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.

12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents
- Unused well strips should be returned to the plate packet and stored at +4°C
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay.

Recommended plate layout

	1	**2**	**3**	**4**
A	B_s	Std 1	Std 5	Sample 1
B	B_s	Std 1	Std 5	Sample 1
C	TA	Std 2	Std 6	Sample 2
D	TA	Std 2	Std 6	Sample 2
E	NSB	Std 3	Std 7	etc
F	NSB	Std 3	Std 7	etc
G	B_0	Std 4	Std 8	
H	B_0	Std 4	Std 8	

Plate layout shows controls, blanks and standards required for each assay. Use additional strips of wells to assay all your samples.

Key:

B_s = Blank; contains substrate only.

TA = Total Activity; contains conjugate (5 µL) and substrate.

NSB = Non-specific binding; contains standard diluent, assay buffer, conjugate and substrate.

B_0 = 0 pg/mL standard; contains standard diluent, conjugate, antibody and substrate

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use**
- **It is recommended to assay all standards, controls and samples in duplicate**
- **Refer to the recommended plate layout in Section 12 before proceeding with the assay.**

13.1 Add 100 µL appropriate diluent* into the NSB (non-specific binding) wells. (*Use the same diluent used to prepare standards in section 10, either Assay Buffer or Tissue Culture Media).

13.2 Add 100 µL appropriate diluent (Assay Buffer or tissue culture media) into the B_0 (0 pg/mL standard) wells.

13.3 Add 100 µL of prepared standards and 100 µL diluted samples to appropriate wells.

13.4 Add 50 µL of Assay Buffer into the NSB wells.

13.5 Add 50 µL of TXB_2 Alkaline Phosphatase Conjugate (blue) into NSB, B_0, standard and sample wells, i.e. not the Total Activity (TA) and B_s wells.

13.6 Add 50 µL of TXB_2 Antibody (yellow) into B_0, standard and sample wells, i.e. not B_s, TA and NSB wells.

Note: Every well used should be green, except the NSB wells which should be blue. B_s and TA wells are empty at this point and have no color.

13.7 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.

13.8 Empty the contents of the wells and wash by adding 400 µL of 1X Wash Buffer to every well. Repeat the wash 2 more times for a total of 3 washes. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

13.9 Add 5 µL of the TXB_2 Alkaline Phosphatase Conjugate to the TA wells only.

13.10 Add 200 µL of the pNpp Substrate solution to every well. Incubate at room temperature for 45 minutes without shaking.

13.11 Add 50 µL Stop Solution into each well. The plate should be read immediately.

13.12 Blank the plate reader against the B_s wells, read the O.D. absorbance 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.

14. CALCULATIONS

14.1 Calculate the average net absorbance measurement (Average Net OD) for each standard and sample by subtracting the average NSB absorbance measurement from the average absorbance measurement (Average OD) for each standard and sample.

Average Net OD = Average Bound OD - Average NSB OD

14.2 Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula

$$\text{Percent Bound} = \frac{\text{Average Net OD}}{\text{Average Net } B_0 \text{ OD}} \times 100$$

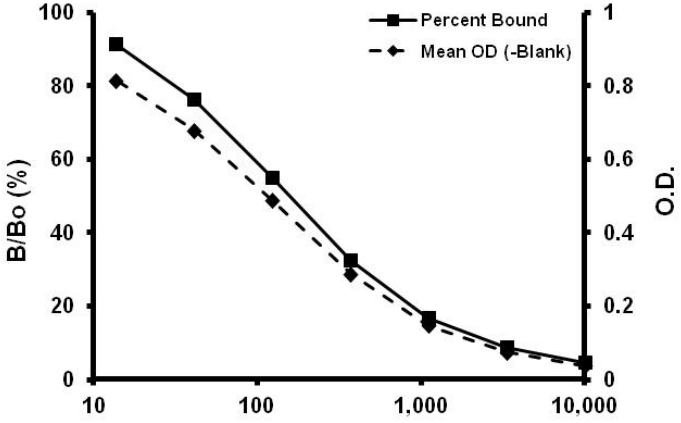
14.3 Plot the Percent Bound (B/B_0) and the net OD versus concentration of TXB_2 for the standards. The concentration of TXB_2 in the unknowns can be determined by interpolation of net OD values.

A four parameter algorithm (4PL) provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Interpolate protein concentrations for unknown samples from the standard curve plotted.

Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

15. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Sample	Mean OD ($-B_s$)	Percent Bound	TXB_2 (pg/mL)
B_s	0.123		
TA	0.693		
NSB	-0.003	0	
B_0	0.891	100	0
S1	0.038	4.6	10,000
S2	0.075	8.7	3,333
S3	0.147	16.8	1,111
S4	0.288	32.4	370.3
S5	0.488	54.9	123.4
S6	0.678	76.2	41.15
S7	0.814	91.3	13.7
Unknown 1	0.154		1,052
Unknown 2	0.410		184

TYPICAL QUALITY CONTROL PARAMETERS

Total Activity Added = 0.570 x 10 = 5.7

%NSB	=	-0.055%
$\%B_0/TA$	=	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

16. TYPICAL SAMPLE VALUES

SENSITIVITY –

Sensitivity was calculated by determining the average optical density bound for 16 wells run as Bo, and comparing to the average optical density for 16 wells run with Standard 7. The detection limit was determined as the concentration of thromboxane B2 measured at 2 standard deviations from the zero along the standard curve was determined to be 10.54 pg/mL.

SAMPLE RECOVERY –

TXB_2 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 TXB_2 was spiked into the undiluted samples, which were then diluted with the kit Assay Buffer. For Human serum and plasma the endogenous levels of TXB_2 in the samples were used. The serum and plasma samples were diluted into the kit Assay Buffer and then assayed in the kit.

Sample Type	Average % 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

LINEARITY OF DILUTION –

A sample containing 3,239 pg/mL TXB_2 was diluted serially 1:2 seven times with 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 1.055 with a correlation coefficient of 0.999.

PRECISION –

Intra-Assay

	TXB_2 (pg/mL)	**%CV**
Low	759	3.6
Medium	1,283	4.0
High	2,605	1.6

Inter-Assay

	TXB_2 (pg/mL)	**%CV**
Low	44	7.6
Medium	371	3.6
High	3,053	6.2

17. ASSAY SPECIFICITY

CROSS REACTIVITY –

The cross reactivities for a number of related eisosanoid compounds was determined by dissolving the cross reactant in assay buffer at concentrations from 500,000 to 13.7 pg/mL. These samples were then measured in the TXB_2 assay, and the measured TXB_2 concentration at 50% B/B_0 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
PGD_2	<0.01
PGE_2	<0.01
PGF_1 alpha	<0.01
PGF_2 alpha	<0.01

18. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed.

19. NOTES

UK, EU and ROW
Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria
Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany
Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain
Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada
Email: ca.technical@abcam.com | Tel: 877-749-8807

China and Asia Pacific
Email: hk.technical@abcam.com | Tel: 400 921 0189 / +86 21 2070 0500

Japan
Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940
www.abcam.com | www.abcam.cn | www.abcam.co.jp