Version 18-10/25

User's Manual

Thromboxane B2 ELISA Kit

REF **DEIA4991**

Σ **96T**

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

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The TXB2 ELISA Kit is a competitive assay that can be used for quantification of TXB2 in urine, serum, tissue culture supernatants, and other sample matrices. The assay has a range from 1.6-1,000 pg/ml and a sensitivity (80% B/B0) of approximately 5 pg/ml.

General Description

Thromboxane A2 (TXA2) is produced from arachidonic acid by many cells and causes irreversible platelet aggregation and contraction of vascular and bronchial smooth muscle. Like most lipid mediators, TXA2 is not a circulating hormone. It is formed in response to local stimuli and exerts its effects within a short distance of its biosynthesis. TXA2 is rapidly hydrolyzed non-enzymatically to form TXB2, which is then quickly metabolized (t1/2 = 5-7 minutes) to urinary metabolites for clearance by the kidneys. Because of the transient nature of this compound it is difficult to accurately measure circulating levels in whole animal experimental models. In fact, it has been shown that plasma and urine levels of TXB2 are primarily due to ex vivo platelet activation and intra-renal production, respectively. Therefore, measurement of TXB2 metabolites, such as 11-dehydro TXB2, in urine and plasma may give more accurate estimates of in vivo TXA2 production. TXB2 measurement is better suited for samples that are unlikely to undergo extensive metabolism such as perfusates, lavage samples, tissue/cell culture, etc.

Principles of Testing

This assay is based on the competition between TXB2 and a TXB2-acetylcholinesterase (AChE) conjugate (TXB2 Tracer) for a limited number of TXB2-specific rabbit antiserum binding sites. Because the concentration of the TXB2 Tracer is held constant while the concentration of TXB2 varies, the amount of TXB2 Tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of TXB2 in the well. This rabbit antiserum-TXB2 (either free or tracer) complex binds to the mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of TXB2 Tracer bound to the well, which is inversely proportional to the amount of free TXB2 present in the well during the incubation; or

Absorbance $\propto$ [Bound TXB2 Tracer] $\propto$ 1/[TXB2]

Reagents And Materials Provided

1. Thromboxane B2 ELISA Antiserum, 1 vial/100 dtn (96-well), 1 vial/500 dtn (480-well)
2. Thromboxane B2 AChE Tracer, 1 vial/100 dtn (96-well), 1 vial/500 dtn (480-well)
3. Thromboxane B2 ELISA Standard, 1 vial (96-well), 1 vial (480-well)
4. ELISA Buffer Concentrate (10X), 2 vials/10 ml (96-well), 4 vials/10 ml (480-well)
5. Wash Buffer Concentrate (400X), 1 vial/5 ml (96-well), 1 vial/12.5 ml (480-well)

6. Polysorbate 20, 1 vial/3 ml (96-well), 1 vial/3 ml (480-well)
7. Mouse Anti-Rabbit IgG Coated Plate, 1 plate (96-well), 5 plates (480-well)
8. 96-Well Cover Sheet, 1 cover (96-well), 5 covers (480-well)
9. Ellman's Reagent, 3 vials/100 dtn (96-well), 6 vials/250 dtn (480-well)
10. ELISA Tracer Dye, 1 vial (96-well), 1 vial (480-well)
11. ELISA Antiserum Dye, 1 vial (96-well), 1 vial (480-well)

Materials Required But Not Supplied

1. A plate reader capable of measuring absorbance between 405-420 nm.
2. Adjustable pipettes and a repeating pipettor.
3. A source of 'UltraPure' water. Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA.
4. Materials used for Sample Preparation.

Storage

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Specimen Collection And Preparation

Sample Preparation

In general, urine, serum, and tissue culture supernatant samples(without FBS) may be diluted with ELISA Buffer and added directly to the assay well. Othersample types may contain contaminants which interfere with the assay.It isbest to check for interference before embarking on a large number of sample measurements. To test for interference, dilute one or two test samples to obtainat least two dilutions of each sample between~5-1,000 pg/ml (i.e.,between20-80%B/B_0).If the two dilutions of the sample show good correlation(differ by 20% or less)in the final calculated TXB2 concentration,purificationis not required.If you do not see good correlation of the different dilutions,purifcation is advised.

General Precautions

1. All samples must be free of organic solvents prior to assay.
2. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at –80°C; they will be stable for approximately 1–2 months.
3. Samples of rabbit origin may contain antibodies which interfere with the assay by binding to the mouse anti-rabbit plate. We recommend that all rabbit samples be purified prior to use in this assay.
4. AEBSF (Pefabloc SC®) and PMSF inhibit AChE. Samples containing these protease inhibitors should not be used in this assay.

Urine

Typically urine samples may be diluted with ELISA Buffer and added directly to the assay. **NOTE:** A substantial portion of urinary TXB2 may be of renal origin.

Serum

Serum should be diluted with ELISA Buffer and assayed without purification.

Tissue Culture Supernatants (without FBS)

Cell culture supernatants without FBS may be assayed directly without purification. If the TXB2 concentration in the medium is high enough to dilute the sample 10-fold with ELISA Buffer, the assay can be performed without any modifications. When assaying less concentrated samples (where samples cannot be diluted with ELISA Buffer), dilute the standard curve in the same culture medium as that used in the experiment. This will ensure that the matrix for the standards is comparable to the samples. We recommend that a standard curve be run first to ensure that the assay will perform in a particular culture medium. **NOTE:** FBS contains high levels of TXB2. If assaying tissue culture supernatants containing FBS, this background TXB2 will need to be subtracted from values obtained for actual samples.

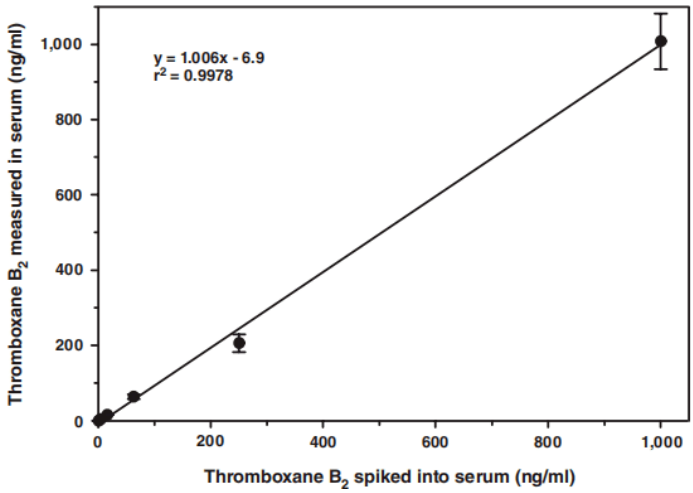


Figure. Recovery of TXB2 from serum

Serum from a healthy donor on an ibuprofen regimen was spiked with TXB2, diluted as described in the Sample Preparation section and analyzed using the TXB2 ELISA Kit. The y-intercept corresponds to the amount of TXB2 in unspiked serum. Error bars represent standard deviations obtained from multiple dilutions of each sample.

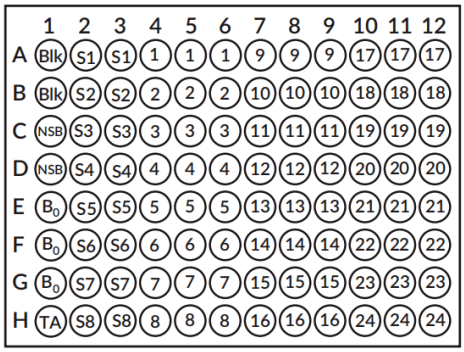
Plate Preparation

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. **NOTE:** If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 4 °C. Be sure the packet is sealed with the desiccant inside.

Each plate or set of strips must contain a minimum of two blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B0), and an eight-point standard curve run in duplicate. **NOTE:** Each assay must contain this minimum configuration in order to ensure accurate and reproducible results. Each sample should be assayed at two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown in Figure. The user may vary the location and type of wells present as

necessary for each particular experiment.



Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8
1-24 - Samples

Reagent Preparation

PRE-ASSAY PREPARATION

NOTE: Water used to prepare all ELISA reagents and buffers must be deionizedand free of trace organic contaminants('UltraPure').Use activated carbon flter cartridges or other organic scavengers. Glass distilled water(even if double distilled), HPLC-grade water,and sterile water(for injections)are not adequate for ELISA.

Buffer Preparation

Store all diluted buffers at 4°C; they will be stable for about two months.

1. ELISA Buffer Preparation

Dilute the contents of one vial of ELISA Buffer Concentrate(10×) with 90 ml of UltraPure water. Be certain to rinse the vial to remove any salts that may have precipitated. **NOTE:** It is normal for the concentrated buffer to contain crystal line salts after thawing. These will completely dissolve upon dilution with water.

2. Wash Buffer Preparation

5 ml vial Wash Buffer Concentrate(400×): Dilute to a total volume of 2 liters with UltraPure water and add1ml of Polysorbate 20.

OR

12. 5 ml vial Wash Buffer Concentrate(400×): Dilute to a total volume of 5 liters with UltraPure water and add 2.5 ml of Polysorbate 20.

Smaller volumes of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Polysorbate 20(0.5 ml/liter of Wash Buffer).

NOTE: Polysorbate 20 is aviscous liquid and cannot be measured bya regular pipette. Apositive displacement pipette or a syringe should be used to deliver small quantities accurately.

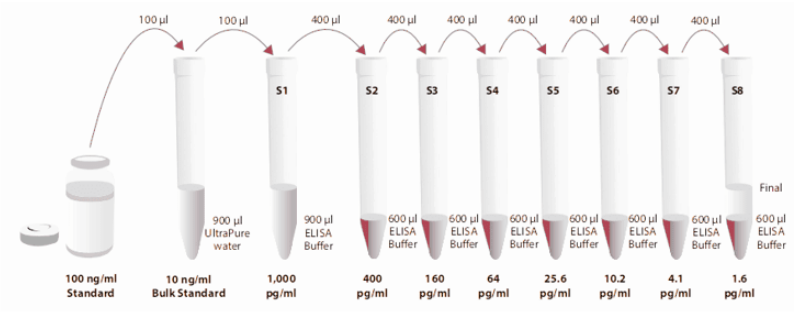
Preparation of Assay-Specific Reagents

1. Thromboxane B2 ELISA Standard

Equilibrate a pipette tip in ethanol by repeatedly filling and expelling the tip with ethanol several times. Using the equilibrated pipette tip, transfer 100 µl of the TXB2 ELISA Standard into a clean test tube, then dilute with 900 µl UltraPure water. The concentration of this solution (the bulk standard) will be 10 ng/ml.

NOTE: If assaying culture medium samples that have not been diluted with ELISA Buffer, culture medium should be used in place of ELISA Buffer for dilution of the standard curve.

To further prepare the standard for use in ELISA: Obtain eight clean test tubes and number them #1 through #8. Aliquot 900 µl ELISA Buffer to tube #1 and 600 µl ELISA Buffer to tubes #2–8. Transfer 100 µl of the bulk standard (10 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 400 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 400 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4–8. The concentrations of these standards are 1,000, 400, 160, 64, 25.6, 10.2, 4.1, and 1.6 pg/ml, respectively. These diluted standards should not be stored for more than 24 hours.



2. Thromboxane B2 AChE Tracer

Reconstitute the TXB2 AChE Tracer as follows:

100 dtn TXB2 AChE Tracer (96-well kit): Reconstitute with 6 ml ELISA Buffer.

OR

500 dtn TXB2 AChE Tracer (480-well kit): Reconstitute with 30 ml ELISA Buffer.

Store the reconstituted TXB2 AChE Tracer at 4 °C (do not freeze!) and use within four weeks. A 20 % surplus of tracer has been included to account for any incidental losses.

Tracer Dye Instructions (optional)

This dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the reconstituted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer or add 300 µl of dye to 30 ml of tracer).

3. Thromboxane B2 ELISA Antiserum

Reconstitute the TXB2 ELISA Antiserum as follows:

100 dtn TXB2 ELISA Antiserum (96-well kit): Reconstitute with 6 ml ELISA Buffer.

OR

500 dtn TXB2 ELISA Antiserum (480-well kit): Reconstitute with 30 ml ELISA Buffer.

Store the reconstituted TXB2 ELISA Antiserum at 4 °C. It will be stable for at least four weeks. A 20 % surplus of antiserum has been included to account for any incidental losses.

Antiserum Dye Instructions (optional)

This dye may be added to the antiserum, if desired, to aid in visualization of antiserum-containing wells. Add the dye to the reconstituted antiserum at a final dilution of 1:100 (add 60 µl of dye to 6 ml antiserum or add 300 µl of dye to 30 ml of antiserum).

Assay Procedure

Pipetting Hints

1. Use different tips to pipette each reagent.
2. Before pipetting each reagent, equilibrate the pipette tip in that reagent (i.e., slowly fill the tip and gently expel the contents, repeat several times).
3. Do not expose the pipette tip to the reagent(s) already in the well.

Addition of the Reagents

1. ELISA Buffer

Add 100 µl ELISA Buffer to NSB wells. Add 50 µl ELISA Buffer to B0 wells. If culture medium was used to dilute the standard curve, substitute 50 µl of culture medium for ELISA Buffer in the NSB and B0 wells (i.e., add 50 µl culture medium to NSB and B0 wells and 50 µl ELISA Buffer to NSB wells).

2. Thromboxane B2 ELISA Standard

Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. Samples

Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. Thromboxane B2 AChE Tracer

Add 50 µl to each well except the TA and the Blk wells.

5. Thromboxane B2 ELISA Antiserum

Add 50 µl to each well except the TA, the NSB, and the Blk wells.

Well-by-Well Pipetting Summary Table:

Well	ELISA Buffer	Standard/Sample	Tracer	Antiserum
Blk	-	-	-	-
TA	-	-	5 µl (at devl. step)	-
NSB	100 µl	-	50 µl	-
B_0	50 µl	-	50 µl	50 µl
Std/Sample	-	50 µl	50 µl	50 µl

Incubation of the Plate

Cover each plate with plastic film and incubate overnight at 4 °C.

Development of the Plate

1. Reconstitute Ellman's Reagent immediately before use (20 ml of reagent is sufficient to develop 100 wells):

   100 dtn vial Ellman's Reagent (96-well kit): Reconstitute with 20 ml of UltraPure water.

   OR

250 dtn vial Ellman's Reagent (480-well kit): Reconstitute with 50 ml of UltraPure water.

NOTE: Reconstituted Ellman's Reagent is unstable and should be used the same day it is prepared; protect the Ellman's Reagent from light when not in use. Extra vials of the reagent have been provided should a plate need to be re-developed or multiple assays run on different days.

2. Empty the wells and rinse five times with Wash Buffer.
3. Add 200 µl of Ellman's Reagent to each well.
4. Dilute the Thromboxane B2 AChE Tracer 1:10 with ELISA Buffer (for example, 50 µl Tracer into 450 µl Assay Buffer). Add 5 µl of tracer to the TA wells.
5. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark. This assay typically develops (i.e., B0 wells ≥0.3 A.U. (blank subtracted)) in 90–120 minutes.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Remove the plate cover being careful to keep Ellman's Reagent from splashing on the cover. **NOTE:** Any loss of Ellman's Reagent will affect the absorbance readings. If Ellman's Reagent is present on the cover, use a pipette to transfer the Ellman's Reagent into the well. If too much Ellman's Reagent has splashed on the cover to easily redistribute back into the wells, wash the plate three times with wash buffer and repeat the development with fresh Ellman's Reagent.
3. Read the plate at a wavelength between 405 and 420 nm. The absorbance may be checked periodically until the B0 wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B0 wells are in the range of 0.3–1.5 A.U. (blank subtracted). If the absorbance of the wells exceeds 2.0, wash the plate, add fresh Ellman's Reagent and let it develop again.

Calculation

ANALYSIS

Many plate readers come with data reduction software that plot data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as either %B/B0 versus log concentration using a four-parameter logistic fit or as logit B/B0 versus log concentration using a linear fit.

Calculations

Preparation of the Data:

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B0 wells.
3. Subtract the NSB average from the B0 average. This is the corrected B0 or corrected maximum binding.
4. Calculate the B/B0 (Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B0 (from Step 3). Repeat for S2–S8 and all sample wells. (To obtain %B/B0 for a logistic four-parameter fit, multiply these values by 100.)

NOTE: The TA values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; the corrected B0 divided by the actual TA (10× measured absorbance) will give the %Bound. This value should closely approximate the %Bound that can be calculated from the Sample Data. Erratic absorbance values and a low (or no) %Bound could indicate the presence of organic solvents in the buffer or other technical problems.

Plot the Standard Curve

Plot %B/B0 for standards S1–S8 versus TXB2 concentration using linear (y) and log (x) axes and perform a 4-parameter logistic fit.

Alternative Plot — The data can also be linearized using a logit transformation. The equation for this conversion is shown below. **NOTE:** Do not use %B/B0 in this calculation.

$$\text{logit}\ (B/B0) = \ln\ [B/B0/(1 - B/B0)]$$

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

Determine the Sample Concentration

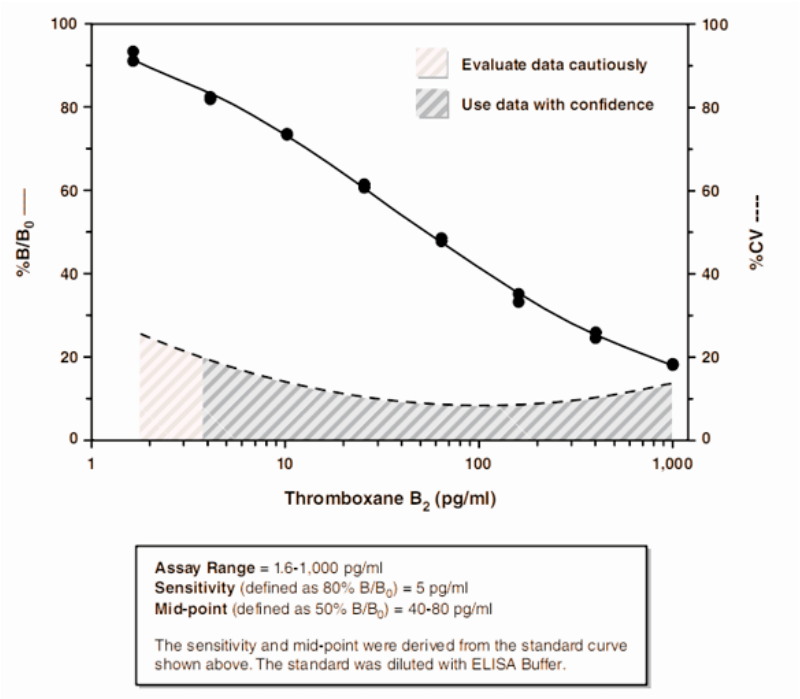
Calculate the B/B0 (or %B/B0) value for each sample. Determine the concentration of each sample using the equation obtained from the standard curve. NOTE: Remember to account for any concentration or dilution of the sample prior to the addition to the well. Samples with %B/B0 values greater than 80 % or less than 20 % should be re-assayed as they generally fall out of the linear range of the standard curve. A 20 % or greater disparity between the apparent concentration of two different dilutions of the same sample indicates interference which could be eliminated by purification.

Typical Standard Curve

The standard curve presented here is an example of the data typically producedwith this kit; however,your results will not be identical to these.You must run a new standard curve. Do not use the data below to determine the values of yoursamples. Your results could differ substantially.

	Raw Data		Average	Corrected
Total Activity	0.523	0.527	0.525	
NSB	0.002	0.001	0.002	
B_0	0.879	0.929		
	0.935	0.971	0.929	0.927

Dose (pg/ml)	Raw Data		Corrected		%B/B_0	
1,000	0.172	0.169	0.170	0.167	18.3	18.0
400	0.242	0.232	0.240	0.230	25.9	24.8
160	0.328	0.309	0.326	0.307	35.1	33.2
64	0.445	0.451	0.443	0.449	47.8	48.5
25.6	0.564	0.572	0.562	0.570	60.6	61.4
10.2	0.684	0.682	0.682	0.680	73.6	73.3
4.1	0.761	0.766	0.759	0.764	81.8	82.4
1.6	0.867	0.846	0.865	0.844	93.3	91.0



Precision

The intra-and inter-assay CVs have been determined at multiple points on thestandard curve.These data are summarized below.

Dose (pg/ml)	%CV* Intra-assay variation
1,000	13.2
400	11.5
160	8.1
64	7.9
25.6	10.8
10.2	13.5
4.1	20.4
1.6	25.8

Table. Intra-assay variation *%CV represents the variation in concentration(not absorbance)as determinedusing a reference standard curve.

Level	Average (ng/ml)	%CV Intra-assay variation	Average (ng/ml)	%CV Inter-assay variation
High	303.3	11.1	326.1	12.9
Medium	81.42	15.3	99.56	12.8
Low	4.666	8.2	4.711	9.9

Detection Range

1.6-1,000 pg/ml

Sensitivity

Sensitivity (80% B/B0): 5 pg/ml.

Specificity

Thromboxane B2: 100%

Thromboxane B3: 33.8%

2, 3-dinor Thromboxane B2: 18.5%

11- dehydro Thromboxane B2: 5.4%

11- dehydro Thromboxane B3: 1.6%

Prostaglandin D2: 0.8%

Prostaglandin F2α: 0.1%

Leukotriene B4: <0.01%

tetranor-PGEM: <0.01%

tetranor-PGFM: <0.01%

Prostaglandin E2: <0.01%

Prostaglandin F1α: <0.01%

2, 3-dinor-6-keto Prostaglandin F1α: <0.01%

6- keto Prostaglandin F1α: <0.01%

13, 4-dihydro-15-keto Prostaglandin F2α: <0.01%