Lipoxin A_4 ELISA Kit

Item No. 590410

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size
490412	Lipoxin A_4 Monoclonal Antibody	1 vial/100 dtn
490410	Lipoxin A_4 AChE Tracer	1 vial/100 dtn
490414	Lipoxin A_4 ELISA Standard	1 vial
400060	ELISA Buffer Concentrate (10X)	2 vials/10 ml
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml
400035	Polysorbate 20	1 vial/3 ml
400050	Ellman's Reagent	3 vials/100 dtn
400040	ELISA Tracer Dye	1 vial
400042	ELISA Antiserum Dye	1 vial
400008/400009	Precoated (Goat Anti-Mouse IgG) ELISA 96-Well Plate	1 plate
400012	96-Well Cover Sheet	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

When compared to quantification by LC/MS or GC/MS, it is not uncommon for immunoassays to report higher analyte concentrations. While LC/MS or GC/MS analyses typically measure only a single compound, antibodies used in immunoassays sometimes recognize not only the target molecule, but also structurally related molecules, including biologically relevant metabolites. In many cases, measurement of both the parent molecule and metabolites is more representative of the overall biological response than is the measurement of a short-lived parent molecule. It is the responsibility of the researcher to understand the limits of both assay systems and to interpret their data accordingly.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

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

Materials Needed But Not Supplied

1. An orbital microplate shaker set at ~500 rpm.
2. A plate reader capable of measuring absorbance at 414 nm.
3. Adjustable pipettes and a repeating pipettor.
4. A source of ultrapure water, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*

INTRODUCTION

Background

Lipoxin A_4 (LXA_4; Item No. 90410) is a part of the specialized pro-resolving mediator (SPM) family of polyunsaturated fatty acid (PUFA) metabolites.[1] It is formed from arachidonic acid through double lipoxygenase-catalyzed reactions initiated by either 5-lipoxygenase (5-LO) followed by 12-LO/15-LO, with a leukotriene A_4 (LTA_4) intermediate, or by 15-LO followed by 5-LO, with 15(S)-HETE and 5(S)-Hp-15(S)-HETE intermediates. The generation of LXA_4 typically requires transcellular metabolism of arachidonic acid.[1,2] For example, LTA_4 that is synthesized in neutrophils by 5-LO is metabolized to LXA_4 in platelets by 12-LO.[2]

LXA_4 is released during the resolution phase of inflammation and binds to the G protein-coupled LXA_4 receptor (ALX)/formyl peptide receptor (FPR2), which is located on leukocytes, and induces cell type-specific signaling.[1] In macrophages, it activates phosphatidylinositol 3-kinase (PI3K) and AKT, stimulates non-phlogistic phagocytosis of apoptotic leukocytes, and inhibits apoptosis to prolong the phagocytotic phase.[3,4] In neutrophils, it inhibits chemotaxis and transmigration and inhibits production of LTB_4 (Item No. 20110).[5]

About This Assay

Cayman's LXA_4 ELISA Kit is a competitive assay which can be used for quantification of LXA_4. This assay has been tested in urine, serum, and plasma. The ELISA typically displays an IC_{50} (50% B/B_0) of approximately 200 pg/ml and a detection limit (80% B/B_0) of approximately 50 pg/ml.

Description of AChE Competitive ELISAs

This assay is based on the competition between LXA_4 and a LXA_4-acetylcholinesterase (AChE) conjugate (LXA_4 AChE Tracer) for a limited amount of LXA_4 Monoclonal Antibody. Because the concentration of the LXA_4 AChE Tracer is held constant while the concentration of LXA_4 varies, the amount of LXA_4 AChE Tracer that is able to bind to the LXA_4 Monoclonal Antibody will be inversely proportional to the concentration of LXA_4 in the well. This antibody-LXA_4 complex binds to goat polyclonal anti-mouse 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 414 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of LXA_4 AChE Tracer bound to the well, which is inversely proportional to the amount of free LXA_4 present in the well during the incubation; or

$$\text{Absorbance} \propto [\text{Bound } LXA_4 \text{ AChE Tracer}] \propto 1/[LXA_4]$$

A schematic of this process is shown in Figure 2, below.

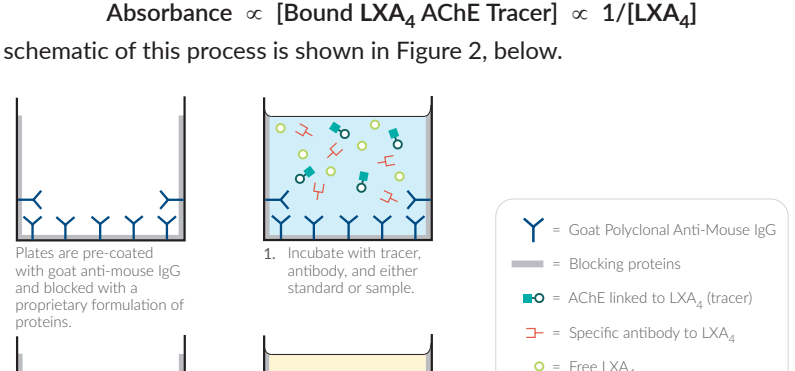


Figure 1. Schematic of the AChE ELISA

Definition of Key Terms

Blank: background absorbance caused by Ellman's Reagent. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including NSB wells.

Total Activity: total enzymatic activity of the AChE-linked tracer.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding.

B_0 (Maximum Binding): maximum amount of the tracer that the antibody can bind in the absence of free analyte.

%B/B_0 (%Bound/Maximum Bound): ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B_0) well.

Standard Curve: a plot of the %B/B_0 values *versus* concentration of a series of wells containing various known amounts of analyte.

Dtn: determination, where one dtn is the amount of reagent used per well.

Cross Reactivity: numerical representation of the relative reactivity of this assay towards structurally related molecules as compared to the primary analyte of interest. Biomolecules that possess similar epitopes to the analyte can compete with the assay tracer for binding to the primary antibody. Substances that are superior to the analyte in displacing the tracer result in a cross reactivity that is greater than 100%. Substances that are inferior to the primary analyte in displacing the tracer result in a cross reactivity that is less than 100%. Cross reactivity is calculated by comparing the mid-point (50% B/B_0) value of the tested molecule to the mid-point (50% B/B_0) value of the primary analyte when each is measured in assay buffer using the following formula:

$$\text{\% Cross Reactivity} = \left[\frac{\text{50\% } B/B_0 \text{ value for the primary analyte}}{\text{50\% } B/B_0 \text{ value for the potential cross reactant}} \right] \text{x } 100\%$$

Lower Limit of Detection (LLOD): the smallest measure that can be detected with reasonable certainty for a given analytical procedure. The LLOD is defined as a point two standard deviations away from the mean zero value.

PRE-ASSAY PREPARATION

Buffer Preparation

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

1. **ELISA Buffer Preparation**

   Dilute the contents of one vial of ELISA Buffer Concentrate (10X) (Item No. 400060) with 90 ml of pure 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 crystalline salts after thawing. These will completely dissolve upon dilution with pure water.*

2. **Wash Buffer Preparation**

   5 ml vial Wash Buffer Concentrate (400X) (Item No. 400062): Dilute to a total volume of 2 liters with pure water and add 1 ml of Polysorbate 20 (Item No. 400035).

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 a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.

Sample Preparation

Testing for Interference

This assay has been tested using human plasma, serum, and urine. Other samples types should be checked for interference to evaluate the need for sample purification before embarking on a large number of sample measurements. To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between approximately 800 pg/ml and 50 pg/ml (i.e., between 20-80% B/B_0, which is the linear portion of the standard curve). The two different dilutions of the sample should show good correlation (differ by 20% or less) in the final calculated LXA_4 concentration.

Urine

Interference in urine is infrequent. However, it is recommended that urine samples be diluted at least 1:2 into ELISA Assay Buffer prior to testing in the assay.

Serum

Interference in serum is infrequent. However, it is recommended that serum samples be diluted at least 1:2 into ELISA Assay Buffer prior to testing in the assay.

Plasma

Interference in plasma is infrequent. However, it is recommended that plasma samples be diluted at least 1:4 into ELISA Assay Buffer prior to testing in the assay.

Sample Matrix Properties

Linearity

Human urine, serum, and plasma samples were spiked with LXA_4 and checked at multiple dilutions using the LXA_4 ELISA Kit. The results are show in the table below.

Dilution Factor	LXA_4 (ng/ml)	Dilutional Linearity (%)
8	4.19	100.0
16	4.04	96.4
32	3.74	89.3

Table 1. Dilutional linearity of spiked human urine in the LXA_4 ELISA Kit.

Dilution Factor	LXA_4 (ng/ml)	Dilutional Linearity (%)
8	5.50	100.0
16	6.12	111.3
32	6.03	110.6
64	5.46	99.3
128	4.97	90.4

Table 2. Dilutional linearity of spiked human serum in the LXA_4 ELISA Kit.

Dilution Factor	LXA_4 (ng/ml)	Dilutional Linearity (%)
4	3.66	100.0
8	3.85	105.2
16	3.47	94.8
32	3.29	89.9

Table 3. Dilutional linearity of spiked human plasma in the LXA_4 ELISA Kit.

Spike and Recovery

Human urine, plasma, and serum were spiked with LXA_4, diluted as described in the **Sample Preparation** section, and analyzed using the LXA_4 ELISA Kit. The results are shown below. The y-intercept corresponds to the amount of endogenous LXA_4 in the sample. The error bars represent standard deviations obtained from multiple dilutions of each sample.

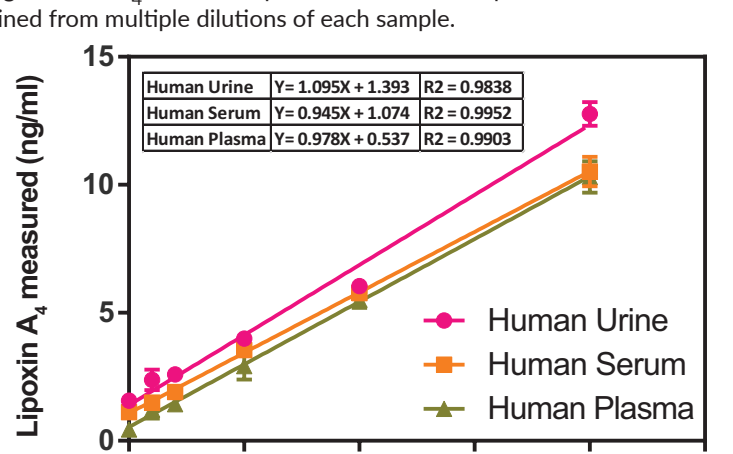


Figure 2. Spike and recovery of Lipoxin A_4 from human urine, serum, and plasma samples

Parallelism

To assess parallelism, human urine, serum, and plasma samples were checked at multiple dilutions using the LXA_4 ELISA Kit. Concentrations were plotted as a function of the sample dilution. The results are shown below. Parallelism of the curves demonstrates that the antigen binding characteristics are similar enough to allow the accurate determination of native analyte levels in diluted human urine, human serum, and human plasma samples.

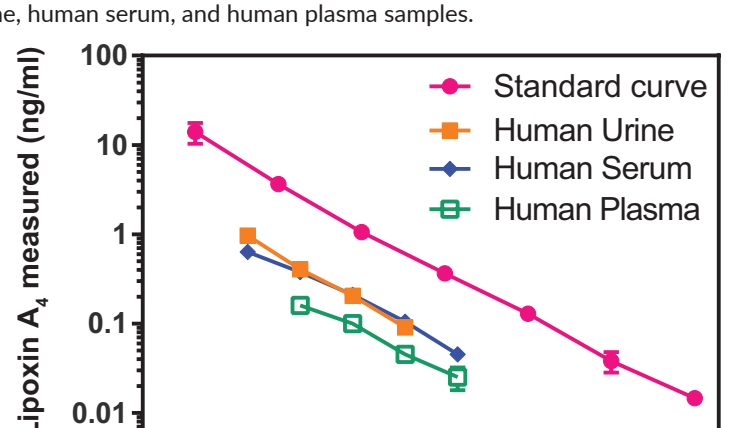


Figure 3. Parallelism of sample matrices in the Lipoxin A_4 ELISA Kit

Preparation of Assay-Specific Reagents

Lipoxin A_4 ELISA Standard

Equilibrate a pipette tip by repeatedly filling and expelling the tip with the LXA_4 Standard (Item No. 490414) several times. Using the equilibrated pipette tip, transfer 100 µl of the LXA_4 Standard into a clean test tube, then dilute with 900 µl pure water. The concentration of this solution (the bulk standard) will be 50 ng/ml.

To prepare the standard for use in the ELISA: obtain eight clean test tubes and number #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 working Standard solution (50 ng/ml) to tube #1 and mix thoroughly. The concentration of this Standard, the first point on the standard curve, will be 5 ng/ml. Serially dilute the standard by removing 400 µl from tube #1 and placing it in tube #2; mix thoroughly. Repeat this process for tubes #3-8.

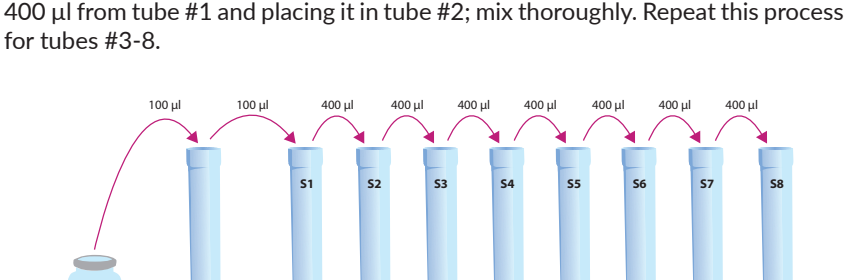


Figure 4. Preparation of the LXA_4 standards

Lipoxin A_4 AChE Tracer

Reconstitute the Lipoxin A_4 AChE Tracer (Item No. 490410) with 6 ml of ELISA Buffer. Store the reconstituted LXA_4 AChE Tracer at 4°C *(do not freeze!)* and use within one week. 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).

Lipoxin A_4 Monoclonal Antibody

Reconstitute the Lipoxin A_4 Monoclonal Antibody (Item No 490412) with 6 ml of ELISA Buffer. Store the reconstituted LXA_4 Monoclonal Antibody at 4°C *(do not freeze!)* and use within one week. A 20% surplus of tracer has been included to account for any incidental losses.

Antibody Dye Instructions (optional)

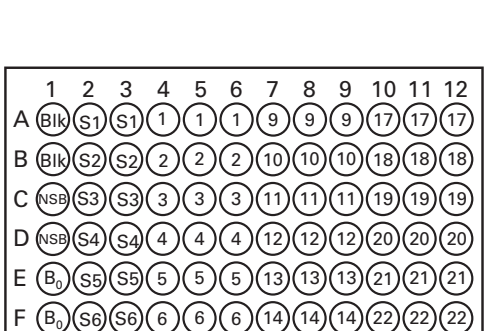
This dye may be added to the antibody, if desired, to aid in visualization of antibody-containing wells. Add the dye to the reconstituted antibody at a final dilution of 1:100 (add 60 µl of dye to 6 ml antibody).

Plate Set Up

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

Each plate or set of strips must contain 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 a minimum of 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 5, below. The user may vary the location and type of wells present as necessary for each particular experiment. We suggest you record the contents of each well on the template sheet provided (see page 27).



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

Figure 5. Sample plate format

Performing the Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- 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).
- Do not expose the pipette tip to the reagent(s) already in the well.

Addition of the Reagents

1. **ELISA Buffer**

   Add 100 µl of ELISA Buffer to Non-Specific Binding (NSB) wells. Add 50 µl ELISA Buffer to Maximum Binding (B_0) wells.

2. **Lipoxin A_4 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 dispense 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. **Lipoxin A_4 AChE Tracer**

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

5. **Lipoxin A_4 ELISA Monoclonal Antibody**

   Add 50 µl to each well *except* the Total Activity (TA), the Non-Specific Binding (NSB), and the Blank (Blk) wells within 15 minutes of addition of the tracer.

Incubation of the Plate

Cover the plate with plastic film (Item No. 400012) and incubate 18 hours at 4°C.

Development of the Plate

1. Reconstitute Ellman's Reagent (Item No. 400050) immediately before use. Reconstitute 100 dtn vial with 20 ml of pure water.
2. Empty the wells and rinse five times with ~300 µl Wash Buffer.
3. Add 200 µl of Ellman's Reagent Solution to each well.
4. Add 5 µl of the reconstituted tracer to the Total Activity 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., B_0 wells ≥0.6 A.U. (blank subtracted)) in 90 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*
3. Read the plate at a wavelength of 414 nm.

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/B_0 *versus* log concentration using a four-parameter logistic fit or as logit B/B_0 *versus* log concentration using a linear fit. *NOTE: Cayman has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysis/elisa) to obtain a free copy of this convenient data analysis tool.*

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 B_0 wells.
3. Subtract the NSB average from the B_0 average. This is the corrected B_0 or corrected maximum binding.
4. Calculate the B/B_0 (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 B_0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain %B/B_0 for a logistic four-parameter fit, multiply these values by 100.)

Plot the Standard Curve

Plot %B/B_0 for standards S1-S8 *versus* LXA_4 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/B_0 in this calculation.*

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

Plot the data as logit (B/B_0) *versus* log concentrations and perform a linear regression fit.

Determine the Sample Concentration

Calculate the B/B_0 (or %B/B_0) value for each sample. Determine the concentration of each sample using the equation obtained from the standard curve plot. *NOTE: Remember to account for any concentration or dilution of the sample prior to the addition to the well.* Samples with %B/B_0 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.

Performance Characteristics

Representative Data

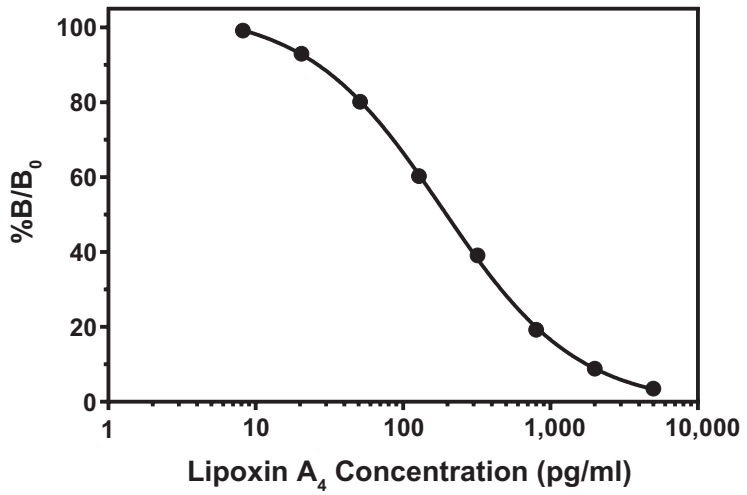
The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples.

Optical Density (O.D.) 414 nm at 90 min.

LXA_4 Standards (pg/ml)	Blank-substracted Absorbance	NSB Corrected Absorbance	%B/B_0	%CV* Intra-assay variation	%CV* Inter-assay variation
NSB	0.003		--	--	--
B_0	0.876	0.873		--	--
5,000	0.034	0.031	3.5	9.8	15.5
2,000	0.080	0.077	8.9	10.5	7.7
800	0.171	0.168	19.2	9.6	11.3
320	0.344	0.341	39.1	9.6	15.8
128	0.529	0.526	60.3	12.3	8.1
51.2	0.702	0.699	80.2	19.5	22.3
20.5	0.814	0.811	93.0	33.0†	32.1†
8.2	0.867	0.864	99.2	67.4†	52.4†
TA	2.234				

Table 4. Intra- and inter-assay variation
*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.

† evaluate data in this range with caution



Assay Range = 8.2-5,000 pg/ml
Sensitivity (defined as 80% B/B_0) = 52.4 pg/ml
Mid-point (defined as 50% B/B_0) = 196.8 pg/ml
Lower Limit of Detection (LLOD) = 39.0 pg/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted in ELISA Buffer.

Figure 6. Typical standard curve

Precision:

Intra-assay precision was determined by analyzing 24 replicates of three matrix controls (human plasma) in a single assay. Inter-assay precision was determined by analyzing replicates of three matrix controls in separate assays spanning across several days.

Matrix Control (pg/ml)	%CV
875.6	3.9
174.0	19.1
62.3	20.4

Table 5. Intra-assay variation

Matrix Control (pg/ml)	%CV
846.5	15.2
184.4	20.5
86.8	20.2

Table 6. Inter-assay variation

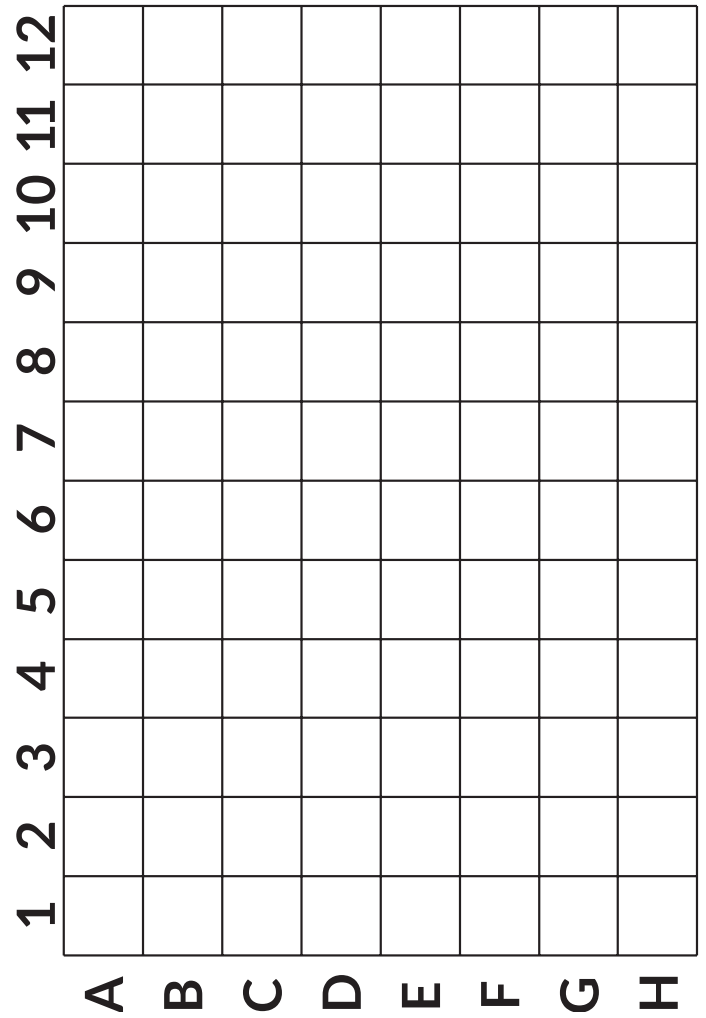
Cross Reactivity:

Compound	Cross Reactivity
Lipoxin A_4 methyl ester	48%
Resolvin D1	37%
15-*epi* Lipoxin A_4	0.74%
Lipoxin B_4	0.57%
Leukotriene B_4	0.01%
Prostaglandin E_2	<0.01%
Arachidonic Acid	<0.01%

Table 7. Cross Reactivity of the LXA_4 ELISA

Lipoxin A_4 Assay Summary					
Procedure	Blk	TA	NSB	B_0	Standards/ Samples
Reconstitute and Mix	Mix all reagents gently				
ELISA Buffer (1X)	-	-	100 µl	50 µl	-
Standards/Samples	-	-	-	-	50 µl
Lipoxin A_4 Tracer	-	-	50 µl	50 µl	50 µl
Lipoxin A_4 Antibody	-	-	-	50 µl	50 µl
Seal	Seal the plate				
Incubate	Incubate 18 hours at 4°C				
Aspirate	Aspirate wells and wash 5 x ~300 µl				
Ellman's Reagent	200 µl	200 µl	200 µl	200 µl	200 µl
Total Activity (TA) - 1X LXA_4 Tracer	-	5 µl	-	-	-
Seal	Seal plate and incubate for 90 min. at room temperature on orbital shaker, protect from light				
Read	Remove plastic seal and read absorbance at 414 nm				

Table 8. Assay Summary



RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique	A. Replace activated carbon filter or change source of UltraPure Water
Poor development (low signal) of standard curve	Standard is degraded	

References

1. Serhan, C.N., Chiang, N., and Van Dyke, T.E. Resolving inflammation: Dual anti-inflammatory and pro-resolution lipid mediators. *Nature Reviews Immunology* **8**, 349-361 (2008).
2. Serhan, C.N. and Sheppard, K.A. Lipoxin formation during human neutrophil-platelet interactions. Evidence for the transformation of leukotriene A_4 by platelet 12-lipoxygenase *in vitro*. *J. Clin. Invest*. **85(3)**, 772-780 (1990).
3. Fierro, I.M., Colgan, S.P., Bernasconi, G., *et al.* Lipoxin A_4 and aspirin-triggered 15-epi-lipoxin A4 inhibit human neutrophil migration: Comparisons between synthetic 15 epimers in chemotaxis and transmigration with microvessel endothelial cells and epithelial cells. *Journal of Immunology* **170**, 2688-2694 (2003).
4. Mitchell, S., Thomas, G., Harvey, K., *et al.* Lipoxins, aspirin-triggered epi-lipoxins, lipoxin stable analogues, and the resolution of inflammation: Stimulation of macrophage phagocytosis of apoptotic neutrophils *in vivo*. *J. Am. Soc. Nephrol.* **13(10)**, 2497-2507 (2002).
5. Godson, C., Mitchell, S., Harvey., K., *et al.* Cutting edge: Lipoxins rapidly stimulate nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages. *J. Immunol.* **164(4)**, 1663-1667 (2000).

NOTES

Warranty and Limitation of Remedy

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