TNF-α (human) ELISA Kit

Item No. 589201

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1180 E. Ellsworth Rd · Ann Arbor, MI · USA

TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size	480 wells Quantity/Size
489202	Anti-TNF-α (human) ELISA Strip Plate	1 plate	5 plates
489200	TNF-α (human) AChE Fab' Conjugate	1 vial/100 dtn	1 vial/500 dtn
489204	TNF-α (human) ELISA Standard	1 vial	1 vial
400060	ELISA Buffer Concentrate (10X)	2 vials/10 ml	4 vials/10 ml
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	1 vial/12.5 ml
400035	Polysorbate 20	1 vial/3 ml	1 vial/3 ml
400012	96-Well Cover Sheet	1 cover	5 covers
400050	Ellman's Reagent	3 vials/100 dtn	6 vials/250 dtn
400100	Human Plasma	1 vial	1 vial
400110	Non-specific Mouse Serum	1 vial	1 vial

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.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's AChE ELISA Kits. This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

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

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

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 at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed 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. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*
4. Materials used for **Sample Preparation** (see page 12).

INTRODUCTION

Background

Tumor necrosis factor-α (TNF-α) is a 17 kDa polypeptide produced primarily by activated monocytes and macrophages. This polypeptide mediates many immune and inflammatory responses, including activation and differentiation of monocytes and macrophages, expression of MHC class I and II, and expression of adhesion molecules on endothelial cells.[1-3] TNF-α is considered to be the primary mediator in many inflammatory conditions including toxic shock and sepsis.[4-5]

About This Assay

Cayman's TNF-α assay is an immunometric (*i.e.*, sandwich) ELISA that permits TNF-α measurements within the range of 3.9-250 pg/ml, typically with a limit of detection (80% B/B_0) of approximately 3.9 pg/ml. This assay allows sensitive, specific analysis of TNF-α in serum or plasma.

Description of AChE Immunometric ELISAs[6]

This immunometric assay is based on a double-antibody 'sandwich' technique. Each well of the microwell plate supplied with the kit has been coated with a monoclonal antibody specific for TNF-α (TNF-α capture antibody). This antibody will bind any TNF-α introduced into the well. An acetylcholinesterase:Fab' Conjugate (AChE:Fab'), which binds selectively to a different epitope on the TNF-α molecule, is also added to the well. When TNF-α (standard or sample) is added to the well, the two antibodies form a 'sandwich' by binding on opposite sides of the TNF-α molecule. The 'sandwiches' are immobilized on the plate so the excess reagents may be washed away. The concentration of the analyte is then determined by measuring the enzymatic activity of the AChE by adding Ellman's Reagent (which contains the substrate for AChE) to each well. The product of the AChE-catalyzed reaction has a distinct yellow color which absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is directly proportional to the amount of bound Conjugate which in turn is proportional to the concentration of the TNF-α.

$$\text{Absorbance} \propto [\text{AChE:Fab' Conjugate}] \propto [\text{TNF-}\alpha]$$

A schematic of this process is shown in Figure 1, below.

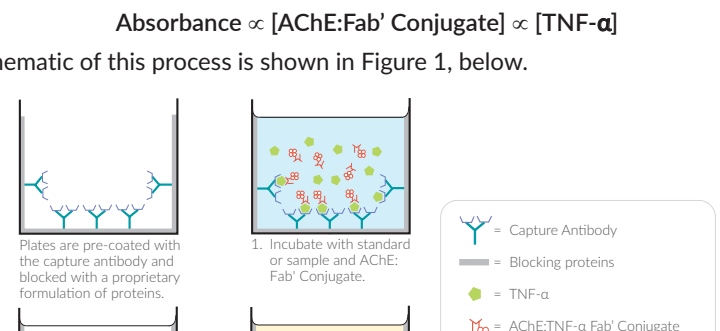


Figure 1. Schematic of the AChE ELISA

Biochemistry of Acetylcholinesterase

The electric organ of the electric eel, *E. electricus*, contains an avid AChE capable of massive catalytic turnover during the generation of its electrochemical discharges. The electric eel AChE has a clover leaf-shaped tertiary structure consisting of a triad of tetramers attached to a collagen-like structural fibril. This stable enzyme is capable of high turnover (64,000 s^{-1}) for the hydrolysis of acetylthiocholine.

A molecule of the analyte covalently attached to a molecule of AChE serves as the tracer in AChE enzyme immunoassays. Quantification of the tracer is achieved by measuring its AChE activity with Ellman's Reagent. This reagent consists of acetylthiocholine and 5,5'-dithio-*bis*-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine (see Figure 2, on page 9). The non-enzymatic reaction of thiocholine with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm (ε = 13,600).

AChE has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. This property of AChE also allows redevelopment of the assay if it is accidentally splashed or spilled. In addition, the enzyme is highly stable under the assay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts or preservatives. Since AChE is stable during the development step, it is unnecessary to use a 'stop' reagent, and the plate may be read whenever it is convenient.

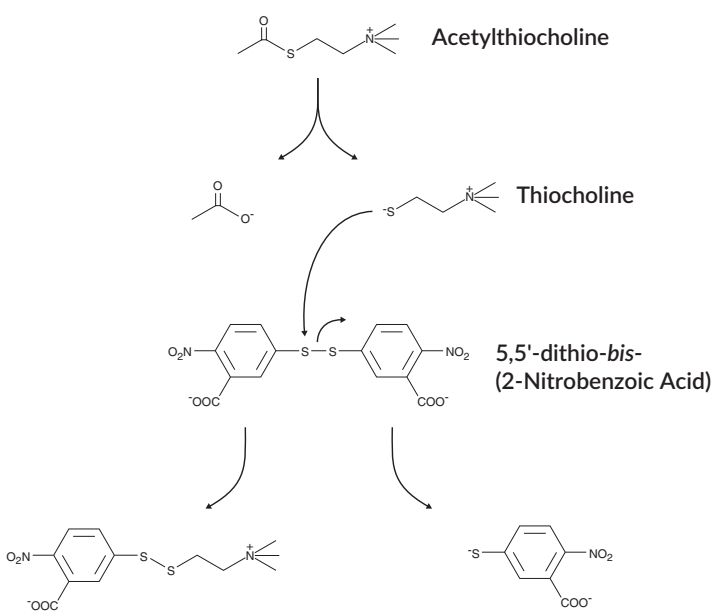


Figure 2. Reaction catalyzed by acetylcholinesterase

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.

Standard Curve: a plot of the absorbance values *versus* concentration of a series of wells containing various known amounts of free analyte.

Sample Matrix Blank (SMB): in order to accurately assay unpurified samples, the standards must be present in the same biological fluid ('matrix') as the samples. One must obtain a supply of this 'matrix' (plasma, synovial fluid, cell culture medium, etc.) which does not contain TNF-α; this is the Sample Matrix Blank. The SMB is used as the diluent for the standard curve. *NOTE: We supply a Human Plasma SMB as part of this kit. If your samples are in any other matrix, sufficient quantities of this matrix must be obtained to use as the diluent for your standard curve.*

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:

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

PRE-ASSAY PREPARATION

NOTE: 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. UltraPure water may be purchased from Cayman (Item No. 400000).

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 (10X) (Item No. 400060) 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 crystalline salts after thawing. These will completely dissolve upon dilution with water.*

2. **Wash Buffer Preparation**

   5 ml vial Wash Buffer Concentrate (400X) (96-well kit; Item No. 400062): Dilute to a total volume of 2 liters with UltraPure water and add 1 ml of Polysorbate 20 (Item No. 400035).

   OR

   12.5 ml vial Wash Buffer Concentrate (400X) (480-well kit; Item No. 400062): Dilute to a total volume of 5 liters with UltraPure water and add 2.5 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

In general, samples can be assayed with no prior purification. If human plasma or synovial fluid is to be tested, one must add the non-specific mouse immunoglobulin (supplied in the kit) to each sample and each point of the standard curve. This will compensate for the effects of human anti-mouse IgG which may be present in the samples. Samples of synovial fluid from patients with rheumatoid arthritis often contain anti-mouse IgM (rheumatoid factors) which can cause erroneously high values. The addition of both the non-specific mouse serum and dithiothreitol (DTT) will alleviate this problem.[6,7] Remember: the standard curve wells must contain the same 'matrix' (including mouse serum and DTT) as the sample wells. Plasma and serum samples should be assayed following a minimum dilution of 1:2 with ELISA Buffer.

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Sample Matrix Blank (SMB) - Human Plasma

The sample matrix blank (SMB) (Item No. 400100) provided with this kit is TNF-α-free human plasma. If this matches your samples, reconstitute the 100 dtn vial with 5 ml of UltraPure water or the 500 dtn vial with 25 ml of UltraPure water. Store this solution at 4°C; it will be stable for approximately two weeks. If your SMB is not human plasma, you must obtain a TNF-α-free SMB that matches your samples. *NOTE: The SMB provided is enough to run one standard curve once reconstituted. More SMB may be purchased by ordering Cayman Human Plasma (Item No. 400100).*

TNF-α (human) ELISA Standard

Dilute the TNF-α (human) ELISA Standard (Item No. 489204) with 2 ml ELISA Buffer. The concentration of this solution will be 5 ng/ml (5,000 pg/ml). Store this solution at 4°C; it will be stable for approximately two weeks. We have included enough TNF-α to run ten standard curves. This surplus should accommodate any experimental design.

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 prepare the standard for use in ELISA: Obtain eight clean test tubes and label them #1 through #8. Aliquot 950 µl SMB to tube #1 and 500 µl SMB to tubes #2-8. Transfer 50 µl of the bulk standard (5 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process until tube #7 has been prepared. Do not add any TNF-α to tube #8. This tube is the zero-point vial, the lowest point on the standard curve. These diluted standards should not be stored for more than 24 hours.

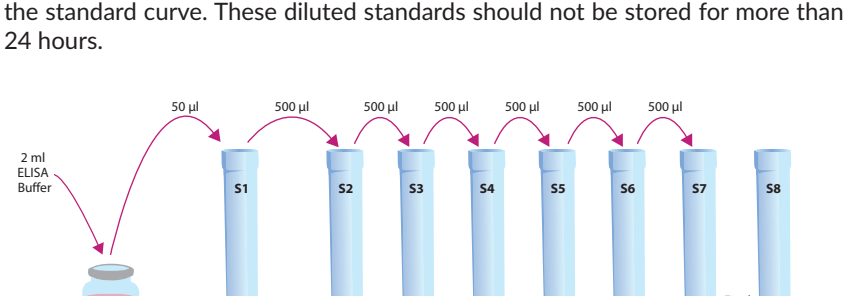


Figure 3. Preparation of the TNF-α standards

Non-specific Mouse Serum

The mouse serum (Item No. 400110) supplied with this kit is to be used when analyzing unpurified plasma, serum, synovial fluid or any other sample that may contain heterophilic antibodies.[7] Reconstitute the mouse serum with 2.5 ml (100 dtn) and 12.5 ml (500 dtn) UltraPure water and store at 4°C. It will be stable for approximately two weeks. A 25 µl aliquot of the mouse serum should be added to each 500 µl aliquot of sample or standard prior to addition to the well. Remember, you must also add the mouse serum to each point of the standard curve (25 µl of mouse serum per 500 µl of standard) to ensure a uniform SMB.

Dithiothreitol - Not included in this kit

If you suspect your samples contain anti-mouse IgM (rheumatoid factors), add 50 µl of 100 mM Dithiothreitol (DTT) to a 500 µl aliquot of each sample. Remember, you must also add the DTT to each point of the standard curve (50 µl of DTT per 500 µl of standard) to ensure a uniform SMB. *NOTE: This is a comparative assay. Although the addition of mouse serum and DTT will change the concentration of your samples and standards, the change will be proportional throughout the assay.*

TNF-α (human) AChE-FAb' Conjugate

Reconstitute the 100 dtn Fab' Conjugate (Item No. 489200) with 10 ml of ELISA Buffer or the 500 dtn Fab' Conjugate with 50 ml of ELISA Buffer. Store the reconstituted Conjugate at 4°C and use within two weeks. A 10% surplus of Conjugate has been included to account for any incidental losses.

Plate Set Up

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 without rinsing 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) 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 4, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see **Analysis**, page 20, for more details). We suggest you record the contents of each well on the template sheet provided (see page 26).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	1	1	1	9	9	9	17	17	17	25
B	S2	S2	2	2	2	10	10	10	18	18	18	25
C	S3	S3	3	3	3	11	11	11	19	19	19	25
D	S4	S4	4	4	4	12	12	12	20	20	20	26
E	S5	S5	5	5	5	13	13	13	21	21	21	26
F	S6	S6	6	6	6	14	14	14	22	22	22	26
G	S7	S7	7	7	7	15	15	15	23	23	23	Blk
H	S8	S8	8	8	8	16	16	16	24	24	24	Blk

Blk - Blank
S1-S8 - Standards 1-8
1-26 - Samples

Figure 4. 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. **TNF-α (human) ELISA Standard**

   Add 100 µl from tube #8 to both of the lowest standard wells (S8). Add 100 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.

2. **Samples**

   Add 100 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).

3. **TNF-α (human) AChE-FAb' Conjugate**

   Add 100 µl to each well *except* the Blk wells.

Well	Standard	Sample	AChE:TNF-α Conjugate
Blk	-	-	-
Standard	100 µl	-	100 µl
Sample	-	100 µl	100 µl

Table 1. Pipetting summary

Incubation of the Plate

Cover each plate with plastic film (Item No. 400012) 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; Item No. 400050): Reconstitute with 20 ml of UltraPure water.

   OR

   250 dtn vial Ellman's Reagent (480-well kit; Item No. 400050): 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 be 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. 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.
5. The plates can be checked periodically over the next few hours. Once the S1 wells seem visibly yellow (0.3 A.U., ~20-30 minutes) it will be possible to determine the concentration of the relatively concentrated samples. Longer development times will be necessary to obtain an accurate plot for the lower range of the standard curve and statistically significant values for sample concentrations near the detection limit of the assay (~3.9 pg/ml). Standard curves at various development times are shown in Figure 5 (see page 19).

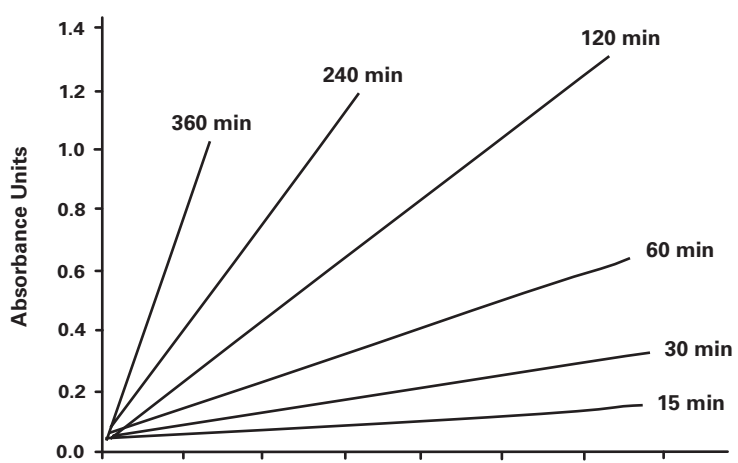


Figure 5. TNF-α Standard curve at various development times

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.

ANALYSIS

Many plate readers come with data reduction software that plots data automatically. Alternatively a spreadsheet program can be used. *NOTE: Cayman Chemical has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysis/immuno) to obtain a free copy of this convenient data analysis tool.*

Calculations

Plot the Standard Curve

Plot absorbance *versus* concentration for standards S1-S8. Construct a best-fit line through the points, including the S8 point.

Determine the Sample Concentration

Use the equation of the line to calculate the concentration of your samples.

$$\text{TNF-α ELISA Concentration (pg/ml)} = \left[\frac{(A_{412}\text{ (sample)} - \text{(y-intercept)})}{\text{Slope}}\right] \times \text{Dilution}$$

Performance Characteristics

Sensitivity:

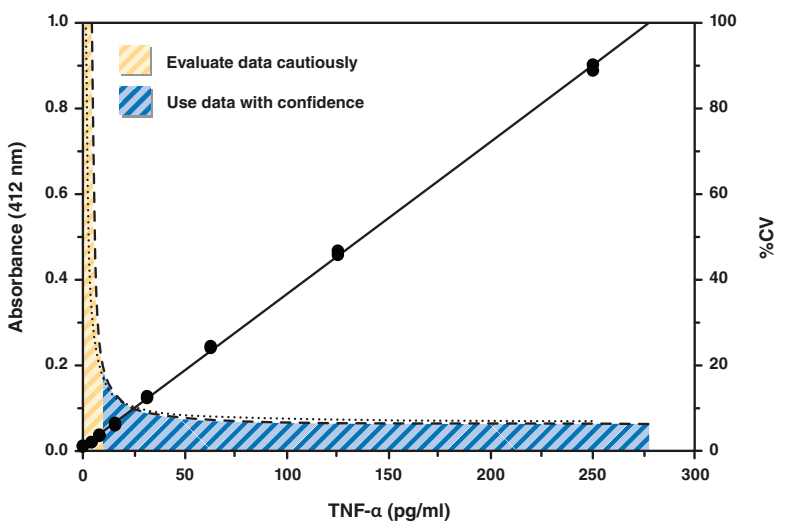
The minimum detectable concentration is 3.9 pg/ml.

Sample 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. Your results could differ substantially.

TNF-α (pg/ml)	Absorbance	
250	0.896	0.902
125	0.462	0.466
62.5	0.242	0.244
31.3	0.126	0.124
15.6	0.063	0.065
7.8	0.037	0.038
3.9	0.020	0.020
0	0.011	0.012

Table 2. Typical results



Assay Range = 3.9-250 pg/ml
LOD = 3.9 pg/ml

The limit of detection (LOD) is defined as the lowest point on the standard curve.The standard was diluted with SMB.

TNF-α Standard curve
TNF-α Intra-assay variation
TNF-α Inter-assay variation

Figure 6. Typical standard curve

Precision:

The intra- and inter-assay CVs have been determined at multiple points on the standard curve. These data are summarized in the graph on page 22 and in the table below.

Dose (pg/ml)	%CV* Intra-assay variation	%CV* Inter-assay variation
250	5.9	9.8
125	5.7	12.1
62.5	6.6	10.3
31.3	9.2	9.0
15.6	15.4	10.4
7.8	27.2	10.4
3.9	111.3	40.6
0	261.9	296.9

Table 3. Intra- and inter-assay variation
*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.

Cross Reactivity:

Compound	Cross Reactivity
TNF-α	100%
TNF-β	<0.01%
TNF-RI	<0.01%
TNF-RII	<0.01%

Table 4. Cross Reactivity of the TNF-α ELISA

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	A. Plate requires additional development time B. Standard was diluted incorrectly C. Standard is degraded	A. Return plate to shaker and re-read later
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present	A. Add mouse serum and DTT to standards and samples B. Purify sample prior to analysis by ELISA[7]
Sample concentrations appear inconsistent with literature values	Matrix for samples and standards are different	A. Use sample matrix for all samples and standards B. Add mouse serum and DTT to standards and samples

References

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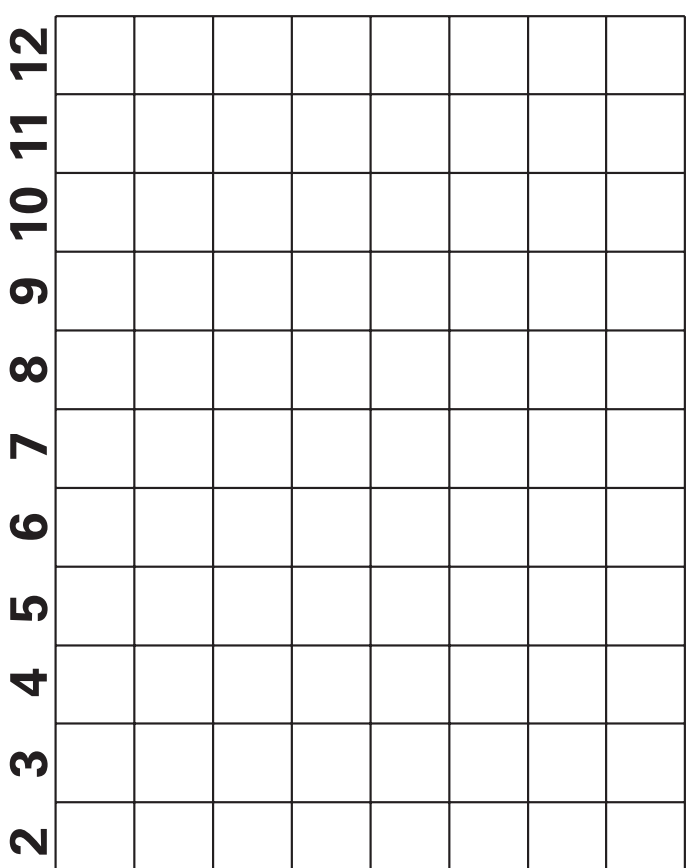
7. Maxey, K.M., Maddipati, K.R., and Birkmeier, J. Interference in enzyme immunoassays. *J. Clin. Immunoassay* **15**, 116-120 (1992).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.