PRODUCT INFORMATION & MANUAL

Mouse TNF-α Valukine™ ELISA

Catalog Number: VAL609

For the quantitative determination of natural and recombinant mouse TNF-α concentrations

For research use only.
Not for diagnostic or therapeutic procedures.

Bio-Techne China Co. Ltd
P: +86 (21) 52380373 P: 8009881270 F: +86 (21) 52381001
info.cn@bio-techne.com

Please refer to the kit label for expiry date.
Novus kits are guaranteed for 3 months from date of receipt

Version 202606.6

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I. BACKGROUND

Tumor necrosis factor alpha (TNF-α), also known as cachectin and TNFSF1A, is the prototypic ligand of the TNF superfamily (1). It is a pleiotropic molecule that plays a central role in inflammation, immune system development, apoptosis, and lipid metabolism (2-5). TNF-α is also involved in a number of pathological conditions including asthma, Crohn's disease, rheumatoid arthritis, neuropathic pain, obesity, type 2 diabetes, septic shock, autoimmunity, and cancer (5-11).

Mouse TNF-α is synthesized as a 26 kDa type II transmembrane protein that consists of a 35 amino acid (aa) cytoplasmic domain, a 21 aa transmembrane segment, and a 179 aa extracellular domain (ECD) (12). Within the ECD mouse TNF-α shares 95% aa sequence identity with rat, and 80% aa identity with canine, equine, feline, human, rabbit, and porcine TNF-α. It is produced by a wide variety of immune, epithelial, endothelial, and tumor cells. TNF-α is assembled intracellularly to form a nonconvalently linked homotrimer which is expressed on the cell surface (13). Cell surface TNF-α can both induce the lysis of tumor cells and virus infected cells, and generate its own downstream cell signaling following ligation by soluble TNF RI (14, 15). Shedding of membrane bound TNF-α by TACE/ADAM17 releases the bioactive cytokine, a 55 kDa soluble trimer containing the TNF-α extracellular domain (16-18).

TNF-α binds the ubiquitous 55-60kDa TNF RI (19, 20) and the hematopoietic cell restricted 78-80 kDa TNF RII (21, 22), both of which are also expressed as homotrimers (1, 23). Both type I and type II receptors bind TNF-α with comparable affinity and can promote NFκB activation (24-27). Only TNF RI, however, contains a cytoplasmic death domain which triggers the activation of apoptosis (3, 28). Soluble forms of both types of receptors are released into human serum and urine and can neutralize the biological activity of TNF (29-31).

II. OVERVIEW

A. PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for mouse TNF-α has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any mouse TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked antibody specific for mouse TNF-α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, TMB substrate (Chromogenic agent) is added to the wells and color develops in proportion to the amount of mouse TNF-α bound in the initial step. The color development is stopped and the intensity of the color is measured.

B. LIMITATIONS OF THE PROCEDURE

- **FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**
- This kit is suitable for cell culture supernate and mouse serum, mouse plasma.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with Calibrator Diluent RD5K/RD6-12 and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

III. ADVANTAGES

A. PRECISION

Intra-assay Precision (Precision within an assay)

Three samples were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples were tested in twenty separate assays to assess inter-assay precision.

CELL CULTURE SUPERNATE ASSAY

	Intra-assay Precision			**Inter-assay Precision**		
Sample	1	2	3	1	2	3
Mean (pg/mL)	54.6	116	405	52.6	138	373
Standard Deviation	1.50	4.51	12.5	4.61	8.49	29.7
CV%	2.7	3.9	3.1	8.8	6.2	8.0

SERUM/PLASMA ASSAY

	Intra-assay Precision			**Inter-assay Precision**		
Sample	1	2	3	1	2	3
Mean (pg/mL)	50.5	134	375	53.0	146	388
Standard Deviation	1.91	4.95	11.5	3.69	8.42	29.7
CV%	3.8	3.7	3.1	7.0	5.8	7.7

B. RECOVERY

The recovery of mouse TNF-α spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range (%)
Cell culture supernates (n=6)	104	94 - 111
Mouse serum (n=4)	94	88 - 106
EDTA plasma (n=4)	95	85 - 103
Heparin plasma (n=4)	92	86 - 98

C. SENSITIVITY

One hundred seventeen assays were evaluated and the minimum detectable dose (MDD) of mouse TNF-α ranged from 0.36-7.21 pg/mL. The mean MDD was 1.88 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

D. CALIBRATION

This immunoassay is calibrated against a recombinant mouse TNF-α produced at R&D Systems®.

The Non WHO Reference Material 88/532 was evaluated in this kit. Each ampule contains a nominal 1 µg of recombinant mouse TNF-α, and was assigned an arbitrary unitage of 200,000 U/ampule. To convert sample values obtained with the Valukine™ Mouse TNF-α kit to approximate NIBSC units, use the equation below:

NIBSC/WHO (88/532) approximate value (U/mL) = 1.8553 x Valukine Mouse TNF-α (pg/mL)

Note: *Based on data generated in March 2017.*

E. LINEARITY

To assess the linearity of the assay, samples were spiked with various concentrations of mouse TNF-α in each matrix, diluted with the appropriate Calibrator Diluent RD5K/RD6-12 and then assayed.

		Cell culture supernates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	106	103	104	105
	Range (%)	102 - 110	90 - 111	102 - 105	98 - 108
1:4	Average % of Expected	105	105	105	110
	Range (%)	98 - 112	92 - 110	101 - 109	105 - 115
1:8	Average % of Expected	100	107	107	112
	Range (%)	89 - 113	99 - 111	101 - 113	110 - 115
1:16	Average % of Expected	105	107	109	109
	Range (%)	99 - 110	97 - 112	101 - 115	101 - 117

F. SAMPLE VALUES

Serum/Plasma - Twenty individual mouse serum and plasma samples were evaluated for levels of mouse TNF-α in this assay. All samples measured less than the lowest Mouse TNF-α Standard, 10.9 pg/mL.

Cell Culture Supernates:

EL-4 mouse lymphoblast cells (2 x 10^5 cells/mL) were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL lipopolysaccharide (LPS) and 100 ng/mL recombinant mouse (rm) IL-10 for 4 days. Aliquots of the cell culture supernates were removed and assayed for levels of mouse TNF-α.

Condition	(pg/mL)
Unstimulated	ND
Stimulated	1220

ND=Non-detectable

RAW 264.7 mouse monocyte/macrophage cells (3.5 x 10^6 cells/mL) were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 5.0 µg/mL LPS and 100 ng/mL rmIL-10 for 7 days. Aliquots of the cell culture supernates were removed and assayed for levels of mouse TNF-α.

Condition	(pg/mL)
Unstimulated	151
Stimulated	9605

Organs from individual mice were removed, rinsed in 1X PBS and kept on ice in tubes containing 1X PBS. Organs were cut into 1-2 mm pieces and homogenized using a tissue homogenizer. Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 1.0 µg/mL LPS for 1 day. Aliquots of the cell culture supernates were removed and assayed for levels of mouse TNF-α.

Tissue Type	Unstimulated (pg/mL)	Stimulated (pg/mL)
Mouse Liver	ND	25.7
Mouse Lung	55.9	97.3
Mouse spleen	ND	34.6

ND=Non-detectable

G. SPECIFICITY

This assay recognizes both natural and recombinant mouse TNF-α.

The following factors were prepared at 100 ng/mL in each calibrator diluent and assayed for cross-reactivity. Preparations of the following factors prepared at 100 ng/mL in a mid-range mouse TNF-α control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse	**Recombinant human**
CD40	TNF-α
CD40 ligand	**Recombinant canine**
Fas	TNF-α
Fas ligand	**Recombinant porcine**
LIF	TNF-α
OPG	
RANK	
RANK-L	
TNF-β	
TRAIL	
TROY	

47% cross-reactivity was observed with recombinant rat TNF-α.

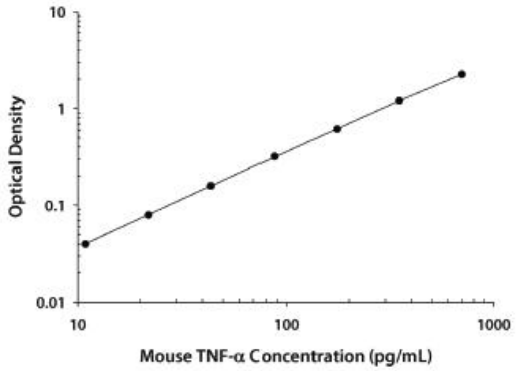
Recombinant mouse TNF RI and TNF RII were found to interfere in this assay at concentrations above 1.25 ng/mL and 12.5 ng/mL, respectively.

IV. EXPERIMENT

EXAMPLE STANDARD

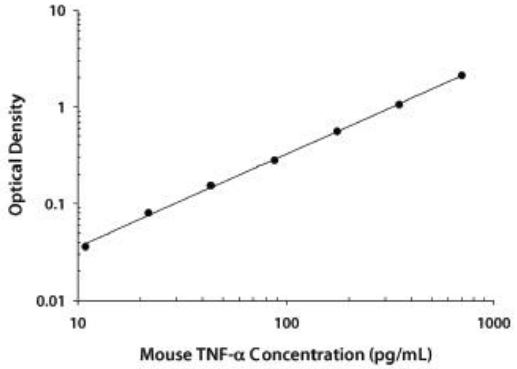
The standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.020 0.021	0.021	—
10.9	0.060 0.061	0.061	0.040
21.9	0.099 0.101	0.100	0.079
43.8	0.179 0.181	0.180	0.159
87.5	0.336 0.344	0.340	0.319
175	0.632 0.637	0.635	0.614
350	1.203 1.242	1.223	1.202
700	2.265 2.281	2.273	2.252

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.013 0.016	0.015	—
10.9	0.051 0.051	0.051	0.036
21.9	0.091 0.099	0.095	0.080
43.8	0.167 0.171	0.169	0.154
87.5	0.285 0.302	0.294	0.279
175	0.571 0.574	0.573	0.558
350	1.057 1.090	1.074	1.059
700	2.111 2.139	2.125	2.110

V. KIT COMPONENTS AND STORAGE

A. MATERIALS PROVIDED

Parts	Description	Size
Mouse TNF-α Microplate	96 well polystyrene microplate (12 strips of 8 wells) coated with antibody against mouse TNF-α	1 plate
Mouse TNF-α Conjugate	Solution of an antibody against mouse TNF-α conjugated to horseradish peroxidase	1 vial
Mouse TNF-α Standard	Recombinant mouse TNF-α in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for the reconstitution volume*	2 vials
Assay Diluent RD1-63	A buffered protein base with preservatives.	1 vial
Calibrator Diluent RD5K	A buffered protein base with preservatives. *For cell culture supernate samples.*	1 vial
Calibrator Diluent RD6-12	A buffered protein base with preservatives. *For serum/plasma samples.*	1 vial
Wash Buffer Concentrate (25×)	A 25× concentrated solution of buffered surfactant	1 vial
TMB Substrate	TMB ELISA Substrate Solution/TMB Substrate Solution	1 vial
Stop Solution	Diluted hydrochloric acid solution	1 vial
Plate Sealers	Adhesive strip	3 strips

B. STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2-8 °C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="8">Opened/ Reconstituted Reagents</td><td>Wash Buffer (1×)</td><td rowspan="4">May be stored for up to 1 month at 2-8 °C.*</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>TMB Substrate</td></tr>
<tr><td>Standard</td><td>Use a new standard for each assay. Discard after use.</td></tr>
<tr><td>Assay Diluent RD1-63</td><td rowspan="3">May be stored for up to 1 month at 2-8 °C.*</td></tr>
<tr><td>Calibrator Diluent RD5K</td></tr>
<tr><td>Calibrator Diluent RD6-12</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil ouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8°C.*</td></tr>
</table>

* Provided this is within the expiration date of the kit.

C. OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.

D. PRECAUTION

- Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.
- The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

VI. PREPARATION

A. SAMPLE COLLECTION AND STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤-20 °C. Avoid repeated freeze-thaw cycles. Samples may require dilution with Calibrator Diluent RD5K.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C Avoid repeated freeze-thaw cycles.Avoid repeated freeze-thaw cycles. Samples may require dilution with Calibrator Diluent RD6-12.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Avoid repeated freeze-thaw cycles. Samples may require dilution with Calibrator Diluent RD6-12.

Note: *Grossly hemolyzed samples are not suitable for use in this assay.*

Citrate plasma has not been validated for use in this assay.

B. REAGENT PREPARATION

Note: *Bring all reagents to room temperature before use.*

Wash Buffer (1×) - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate (25×) into deionized or distilled water to prepare 500 mL of Wash Buffer (1×).

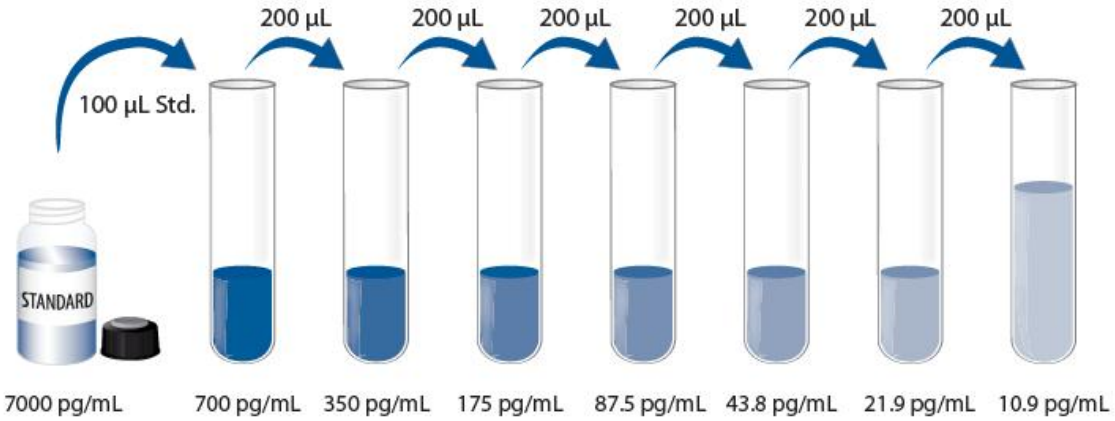
Mouse TNF-α Standard - Refer to the vial label for the reconstitution volume*.

Reconstitute the Mouse TNF-α Standard with deionized or distilled water. This reconstitution produces a stock solution of 7000 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

*If you have any question, please contact our Technical Support.

Pipette 900 µL of Calibrator Diluent RD5K (*for cell culture supernate samples*) or

Calibrator Diluent RD6-12 (*for serum/plasma samples*) into the 700 pg/mL tube. Pipette 200 µL of appropriate Calibrator Diluent into each remaining tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The standard 700 pg/mL serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).



C. TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- It is recommended that the samples be pipetted within 15 minutes.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- TMB Substrate should remain colorless until added to the plate. Keep TMB Substrate protected from light. TMB Substrate should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the TMB Substrate. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the TMB Substrate.

VII. ASSAY PROCEDURE

Note: *Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.*

1. Prepare all reagents, samples and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1-63 to each well.
4. Add 50 µL of Standard and sample per well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. **Incubate for 2 hours at room temperature**. A plate layout is provided for a record of standards and samples assayed.
5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 100 µL of mouse TNF-α conjugate to each well. Cover with a new adhesive strip. **Incubate for 2 hours at room temperature**.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of TMB Substrate to each well. **Incubate for 30 minutes at room temperature**. **Protect from light**.
9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 10 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

11. CALCULATION OF RESULTS.

Average the duplicate readings for each standard and sample and subtract the average zero standard optical density (O.D.).Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the mouse TNF-α concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

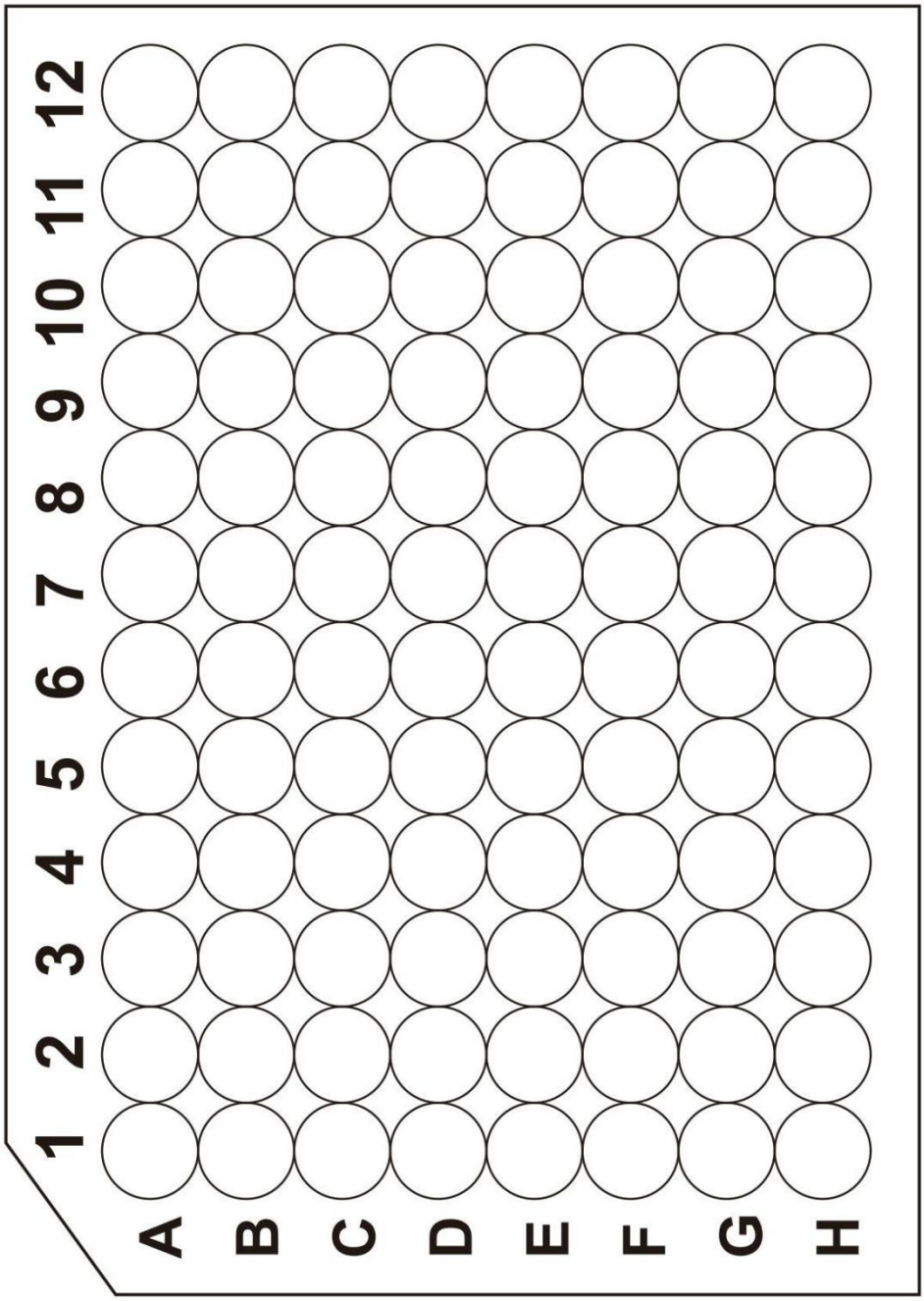
If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



产品信息及操作手册

小鼠 TNF-α Valukine™ ELISA 试剂盒

目录号：VAL609

适用于定量检测天然和重组小鼠 TNF-α的浓度

科研专用，不可用于临床诊断

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版本号 202606.6

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I. 背景

肿瘤坏死因子alpha (TNF-α，又称为cachectin和TNFSF1A)，是肿瘤坏死因子超家族的原型配体（1）。它是一个多效因子，在炎症反应、免疫系统发育、细胞凋亡和类脂物代谢中起到的一个中心作用（2-5）。TNF-α也参与到许多病理过程，包括哮喘、克罗恩病、类风湿关节炎、神经性疼痛、肥胖症、II型糖尿病、感染性休克、自身免疫和癌症（5-11）。

小鼠TNF-α是一个26 kDa的II型跨膜蛋白，由一个35个氨基酸（aa）胞内域、一个21 aa 跨膜段和一个179 aa的胞外域（ECD）组成（12）。在ECD区，小鼠TNF-α和大鼠有95%氨基酸序列同源性，与犬、马、猫，人、兔和猪有80%的氨基酸序列同源性。它可以有多种不同细胞，如免疫细胞、上皮细胞、内皮细胞、肿瘤细胞表达产生。TNF-α在细胞内装配组成非共价键相连的同源三聚体，然后在细胞表面表达（13）。细胞表面的TNF-α能诱导肿瘤细胞和病毒感染细胞的裂解，并与可溶性TNF RI结合，产生下游细胞的跨膜转运信号（14, 15）。TACE/ADMA17可引起含有TNF-α细胞膜的脱落，从而释放具有活性的TNF-α细胞因子，它由TNF-α胞外可溶性结构构成的三聚体，分子量为55 kDa (16-18)。

TNF-α有两个受体：TNF RI和TNF RII。TNF RI分子量为55-60 kDa，表达广泛(19, 20)； TNF RII分子量为78-80 kDa，仅限于造血细胞表达(21, 22)。两者都是以同源三聚体形式表达（1, 23）；TNF-α与TNF RI和TNF RII结合的亲和力相似，可促进NKκβ的激活（24-27）。然而，仅有TNF RI具有胞内死亡结构域，能引发细胞凋亡（3, 28）。这两种可溶性受体都被释放到人的血清和尿液中，并能中和TNF的活性（29-31）。

II. 概述

A. 检测原理

本实验采用双抗体夹心ELISA法。抗小鼠TNF-α抗体包被于微孔板上，样品和标准品中的小鼠TNF-α会与固定在板上的抗体结合，游离的成分被洗去；加入辣根过氧化酶标记的抗小鼠TNF-α抗体进行孵育。洗涤去除未结合的试剂后；加入TMB底物溶液（显色剂），溶液颜色与结合的目标蛋白成正比；加入终止液；用酶标仪测定吸光度。

B. 检测局限

- **仅供科研使用，不可用于体外诊断；**
- 该试剂盒适用于细胞培养上清样本和小鼠血清，小鼠血浆样本；
- 请在试剂盒有效期内使用；
- 不同试剂盒及不同批号试剂盒的组分不能混用；
- 样本值若大于标准曲线的最高值，应将样本用标准品稀释液 RD5K/RD6-12稀释后重新检测；
- 检测结果的不同可由多种因素引起，包括实验人员的操作、移液器的使用方式、洗板技术、反应时间或温度、试剂盒的效期等。

III. 优势

A. 精确度

板内精确度（同一板内不同孔间的精确度）

已知浓度的三个样本，在同一板内分别检测 20 次，以确定板内精确度。

板间精确度（不同板之间的精确度）

已知浓度的三个样本，在不同板间分别检测 20 次，以确定板间精确度。

细胞培养上清

	板内精确度			板间精确度		
样本	1	2	3	1	2	3
平均值(pg/mL)	54.6	116	405	52.6	138	373
标准差	1.50	4.51	12.5	4.61	8.49	29.7
CV%	2.7	3.9	3.1	8.8	6.2	8.0

血清/血浆

	板内精确度			板间精确度		
样本	1	2	3	1	2	3
平均值(pg/mL)	50.5	134	375	53.0	146	388
标准差	1.91	4.95	11.5	3.69	8.42	29.7
CV%	3.8	3.7	3.1	7.0	5.8	7.7

B. 回收率

在不同样本中掺入三个不同水平的小鼠 TNF-α，测定其回收率。

样品类型	平均回收率（%）	范围（%）
细胞培养上清(n=6)	104	94 - 111
小鼠血清(n=4)	94	88 - 106
EDTA血浆(n=4)	95	85 - 103
肝素血浆(n=4)	92	86 - 98

C. 灵敏度

共评估了 117 次测定，小鼠 TNF-α的最低可测剂量（MDD）范围为 0.36~7.21 pg/mL，平均 MDD 为 1.88 pg/mL。

MDD 是根据 20 个重复的零标准品孔的吸光度值的平均值加两倍标准差计算得到的相对应浓度。

D. 校正

此 ELISA 试剂盒经 R&D Systems®生产的重组小鼠 TNF-α蛋白所校正。

本试剂盒对非 WHO 参考物质 88/532 进行了评估。每支安瓿瓶中含有标称 1 µg 的重组小鼠 TNF-α，并被赋予 200,000 U/安瓿的任意单位值。要将使用 Valukine™小鼠 TNF-α试剂盒获得的样本值转换为近似的 NIBSC 单位，请使用以下公式：

NIBSC/WHO (88/532)近似值(U/mL) = 1.8553 × Valukine 小鼠 TNF-α (pg/mL)

注意：*基于 2017 年 3 月生成的数据。*

E. 线性

不同的样本中含有或掺入不同浓度的小鼠TNF-α，然后用相应的标准品稀释液RD5K/RD6-12将样本稀释到检测范围内，测定其线性。		**细胞培养上清（n=4）**	**血清（n=4）**	**EDTA血浆（n=4）**	**肝素血浆（n=4）**
1:2	平均值/期待值（%）	106	103	104	105
	范围（%）	102 - 110	90 - 111	102 - 105	98 - 108
1:4	平均值/期待值（%）	105	105	105	110
	范围（%）	98 - 112	92 - 110	101 - 109	105 - 115
1:8	平均值/期待值（%）	100	107	107	112
	范围（%）	89 - 113	99 - 111	101 - 113	110 - 115
1:16	平均值/期待值（%）	105	107	109	109
	范围（%）	99 - 110	97 - 112	101 - 115	101 - 117

F. 样本预值

血清/血浆 - 在本检测中，对 20 份单独的小鼠血清和血浆样本进行了小鼠 TNF-α水平的评估。所有样本的测得值均低于小鼠 TNF-α最低标准品（10.9 pg/mL）。

细胞培养上清:

将 EL-4 小鼠淋巴母细胞（2 × 10^5个细胞/mL）培养于 DMEM 培养基中，该培养基添加了 10%胎牛血清、2 mM L-谷氨酰胺、100 U/mL 青霉素和 100 µg/mL 硫酸链霉素。细胞分别在未刺激条件下，或在 10 µg/mL 脂多糖（LPS）和 100 ng/mL 重组小鼠（rm）IL-10 刺激条件下培养 4 天。收集细胞培养上清液的分装样品，并检测其中的小鼠 TNF-α水平。

条件	**（pg/mL）**
不刺激	ND
刺激	1220

ND=未检出

将RAW 264.7小鼠单核/巨噬细胞（3.5 × 10^6 细胞/mL）培养于DMEM培养基中，该培养基添加了10%胎牛血清、2 mM L-谷氨酰胺、100 U/mL青霉素和100 µg/mL 硫酸链霉素。细胞分别在未刺激条件下，或在5.0 µg/mL脂多糖（LPS）和100 ng/mL重组小鼠（rm）IL-10刺激条件下培养7天。收集细胞培养上清液的分装样品，并检测其中的小鼠TNF-α水平。

条件	**（pg/mL）**
不刺激	151
刺激	9605

从个体小鼠身上取出器官，在1× PBS中冲洗，并置于含有1× PBS的试管中冰上保存。将器官切成1-2毫米的小块，使用组织匀浆器进行匀浆。细胞培养于添加了10%胎牛血清、2 mM L-谷氨酰胺、100 U/mL青霉素和100 µg/mL硫酸链霉素的RPMI 1640培养基中。细胞分别在未刺激条件下或在1.0 µg/mL LPS刺激条件下培养1天。收集细胞培养上清液的分装样品，并检测其中的小鼠TNF-α水平。

组织类型	未刺激（pg/mL）	刺激（pg/mL）
小鼠肝脏	ND	25.7
小鼠肺	55.9	97.3
小鼠脾脏	ND	34.6

ND=未检出

G. 特异性

此 ELISA 法可检测天然及重组小鼠 TNF-α蛋白。

将以下因子用不同的标准品稀释液配制成 100ng/mL 的浓度来检测与小鼠 TNF-α的交叉反应。将 100 ng/mL 的干扰因子掺入中间范围的重组小鼠 TNF-α标准品中，以此来检测对小鼠 TNF-α的干扰。没有观察到明显的交叉反应或干扰。

Recombinant mouse	**Other Recombinant**
CD40	TNF-α
CD40 ligand	**Recombinant canine**
Fas	TNF-α
Fas ligand	**Recombinant porcine**
LIF	TNF-α
OPG	
RANK	
RANK-L	
TNF-β	
TRAIL	
TROY	

与重组大鼠TNF-α有47%的交叉反应。

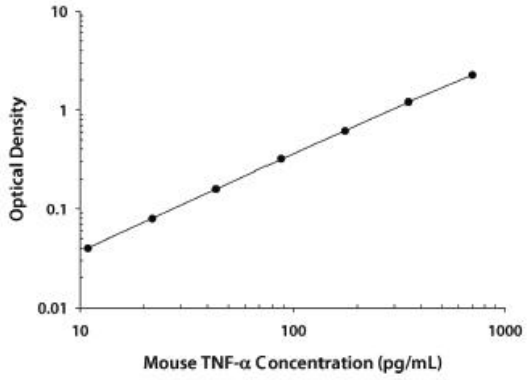
重组小鼠TNF RI和TNF RII在浓度分别大于1.25ng/mL和12.5ng/mL时，会干扰TNF-α的检测。

IV. 实验

标准曲线实例

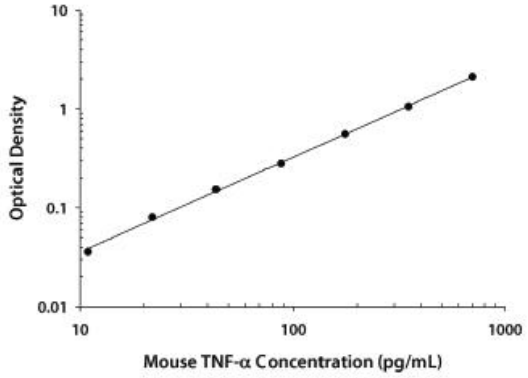
该标准曲线数据仅供参考，每次实验应绘制其对应的标准曲线。

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.020 0.021	0.021	—
10.9	0.060 0.061	0.061	0.040
21.9	0.099 0.101	0.100	0.079
43.8	0.179 0.181	0.180	0.159
87.5	0.336 0.344	0.340	0.319
175	0.632 0.637	0.635	0.614
350	1.203 1.242	1.223	1.202
700	2.265 2.281	2.273	2.252

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.013 0.016	0.015	—
10.9	0.051 0.051	0.051	0.036
21.9	0.091 0.099	0.095	0.080
43.8	0.167 0.171	0.169	0.154
87.5	0.285 0.302	0.294	0.279
175	0.571 0.574	0.573	0.558
350	1.057 1.090	1.074	1.059
700	2.111 2.139	2.125	2.110

V. 试剂盒组成及储存

A. 试剂盒组成

组成	描述	规格
Mouse TNF-α Microplate	包被抗小鼠TNF-α抗体的 96 孔聚苯乙烯板，8 孔×12 条	1 块板
Mouse TNF-α Conjugate	酶标检测抗小鼠TNF-α抗体	1 瓶
Mouse TNF-α Standard	小鼠TNF-α标准品（冻干）溶于含防腐剂的缓冲蛋白基质中。*参考瓶身标签进行重溶*	2 瓶
Assay Diluent RD1-63	含防腐剂的缓冲蛋白基质。	1 瓶
Calibrator Diluent RD5K	含防腐剂的缓冲蛋白基质。*用于细胞培养上清样本。*	1 瓶
Calibrator Diluent RD6-12	含防腐剂的缓冲蛋白基质。*用于血清/血浆样本。*	1 瓶
Wash Buffer Concentrate（25×）	浓缩洗涤缓冲液（25×）	1 瓶
TMB Substrate	TMB ELISA 底物溶液 /TMB 底物溶液	1 瓶
Stop Solution	终止液	1 瓶
Plate Sealers	封板膜	3张

B. 试剂盒储存

<table>
<tr><td>未开封试剂盒</td><td colspan="2">2-8℃储存；请在试剂盒有效期内使用</td></tr>
<tr><td rowspan="8">已打开，稀释或重溶的试剂</td><td>洗涤液（1×）</td><td rowspan="4">2-8℃储存，30天*。</td></tr>
<tr><td>终止液</td></tr>
<tr><td>酶标检测抗体</td></tr>
<tr><td>TMB底物溶液</td></tr>
<tr><td>标准品</td><td>每次使用前新鲜配制。使用后丢弃。</td></tr>
<tr><td>检测液RD1-63</td><td rowspan="3">2-8℃储存，最多 30 天*</td></tr>
<tr><td>标准品稀释液RD5K</td></tr>
<tr><td>标准品稀释液RD6-12</td></tr>
<tr><td>包被的微孔板条</td><td>将未用的板条放回带有干燥剂的铝箔袋内，密封；2-8℃储存，30天*。</td></tr>
</table>

*必须在试剂盒有效期内。

C. 实验所需自备试验器材

- 酶标仪（可测量450 nm检测波长的吸收值及540 nm或570 nm校正波长的吸收值）；
- 高精度加液器及一次性吸头；
- 蒸馏水或去离子水；
- 洗瓶（喷瓶）、多通道洗板器或自动洗板机；
- 500 mL量筒；

D. 注意事项

- 试剂盒中的一些组分含有防腐剂，可能引起皮肤过敏反应，避免吸入。
- 试剂盒中的终止液是酸性溶液，使用时请做好眼睛、手、面部及衣服的防护。

VI. 实验前准备

A. 样品收集及储存

以下样本采集和储存条件仅供一般性参考。样本稳定性尚未经过评估。

细胞培养上清液：颗粒物应离心去除；立刻检测样本。样本收集后若不及时检测，需按一次使用量分装，冻存于≤-20℃冰箱内，避免反复冻融。样本可能需要用标准品稀释液RD5K稀释。

血清样本：让血样在室温下凝固2小时，然后2000 x g离心20分钟。吸取血清样本之后即刻用于检测，或者分装，≤-20℃贮存备用。避免反复冻融。样本可能需要用标准品稀释液RD6-12稀释。

血浆：使用EDTA或肝素作为抗凝剂采集血浆。采集后30分钟内，以2000 x g离心20分钟。吸取血浆样本之后即刻用于检测，于≤-20℃条件下。避免反复冻融。样本可能需要用标准品稀释液RD6-12稀释。

注意：*明显溶血的样本不适用于本检测。*

枸橼酸盐血浆尚未经验证可用于本检测。

B. 检测前准备工作

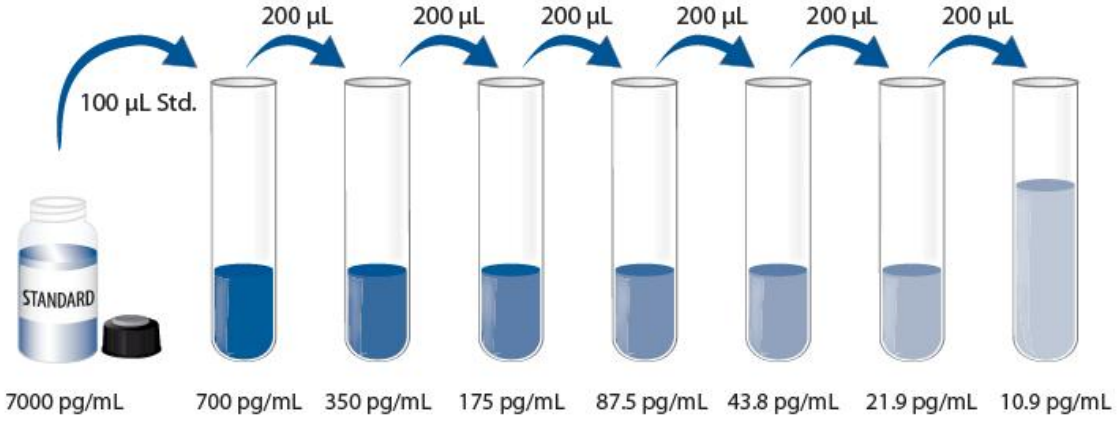
使用前请将所有试剂放置于室温。

洗涤液（1×）：从冰箱中取出的浓缩洗涤液可能有结晶，属于正常现象；放置室温，轻摇混匀，待结晶完全溶解后再配制洗涤液。可将20 mL浓缩洗涤液（25×）用蒸馏水或去离子水稀释配制成 500mL工作浓度的洗涤液（1×）。

小鼠TNF-α标准品：参照标准品瓶身注明的方式重溶冻干标准品*。使用去离子水或蒸馏水复溶小鼠TNF-α标准品。复溶后得到浓度为7000 pg/mL的储备液。在进行稀释前，请让标准品至少静置5分钟，并轻轻混合。

*如有疑问，请咨询我们的技术支持。

在700 pg/mL的稀释管中加入900 µL标准品稀释液RD5K（*用于细胞培养上清样本*）或标准品稀释液RD6-12（*用于血清/血浆样本*），其余每个稀释管中加入200 µL相应的标准品稀释液。将标准品母液参照下图做系列稀释，每管须充分混匀后再移液到下一管。700 pg/mL管作标准曲线最高点，相应的标准品稀释液可用作标准曲线零点（0 pg/mL）。



C. 技术小提示

- 当混合或重溶蛋白液时，尽量避免起沫；
- 为了避免交叉污染，配制不同浓度标准品、上样、加不同试剂都需要更换枪头。另外不同试剂请分别使用不同的移液槽；
- 建议15分钟内完成一块板的上样；
- 每次孵育时，正确使用封板膜可保证结果的准确性；
- TMB底物溶液在上板前应为无色，请避光保存；加入微孔板后，将由无色变成不同深度的蓝色；
- 终止液上板顺序应同TMB底物溶液上板顺序一致；加入终止液后，孔内颜色由蓝变黄；若孔内有绿色，则表明孔内液体未混匀请充分混合。

VII.操作步骤

使用前请将所有试剂和样本放置于室温，建议所有的实验样本和标准品做复孔检测。

1. 按照上一节的说明，准备好所有需要的试剂、样品和标准品；
2. 从已平衡至室温的密封袋中取出微孔板，未用的板条请放回铝箔袋内，重新封口；
3. 在每个微孔中加入50 µL检测液RD1-63；
4. 分别将不同浓度标准品和实验样本加入相应孔中，每孔 50 µL。轻轻敲击板框 1 分钟以混合均匀。用封板膜封住反应孔，**室温孵育 2 小时**。说明书提供了一张 96 孔模板图，可用于记录标准品和试验样本的板内位置；
5. 将板内液体吸去，使用洗瓶、多通道洗板器或自动洗板机洗板。每孔加洗涤液 400 µL，然后将板内洗涤液吸去。重复操作4次，共洗5次。每次洗板尽量吸去残留液体会有助于得到好的实验结果。最后一次洗板结束，请将板内所有液体吸干或将板倒置，在吸水纸拍干所有残留液体；
6. 在每个微孔内加入100 µL酶标检测抗体。用封板膜封住反应孔，**室温孵育2小时**；
7. 重复第5步洗板操作；
8. 在每个微孔内加入100 µL TMB底物溶液，**室温孵育30分钟**。**注意避光**；
9. 在每个微孔内加入100 µL终止液，请轻拍微孔板，使溶液混合均匀；
10. 加入终止液后10分钟内，使用酶标仪测量450 nm的吸光度值，设定540 nm或570 nm作为校正波长。如果波长校正不可用，以450 nm的读数减去540 nm或570 nm的读数。这种减法将校正酶标板上的光学缺陷。没有校正而直接在450 nm处进行的读数可能会更高且更不准确；
11. **计算结果：**将每个标准品和样品的复孔吸光值取平均值，然后减去零标准品平均OD值（O.D.），使用计算机软件作四参数逻辑（4-PL）曲线拟合创建标准曲线。另一替代方法是，通过绘制y轴上每个标准品的平均吸光值与x轴上的浓度来构建标准曲线，并通过图上的点绘制最佳拟合曲线。数据可以通过绘制小鼠TNF-α浓度的对数与O.D.的对数来线性化，并且最佳拟合线可以通过回归分析来确定。该程序将产生足够但不太精确的数据拟合。

    如果样品被稀释，从标准曲线读取的浓度必须乘以稀释倍数。

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