PRODUCT INFORMATION & MANUAL

Human TNF-α Valukine™ ELISA

Catalog Number: VAL105G

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

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

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

Version 202605.6

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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).

Human 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 177 aa extracellular domain (ECD) (12, 13). Within the ECD human TNF-α shares 97% aa sequence identity with rhesus monkey, and 71% - 92% aa identity with bovine, canine, cotton rat, equine, feline, mouse, porcine, and rat 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 (14). 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 (15, 16). Shedding of membrane bound TNF-α by TACE/ADAM17 releases the bioactive cytokine, a 55 kDa soluble trimer of the TNF-α extracellular domain (17-19).

TNF-α binds the ubiquitous 55-60 kDa TNF RI (20, 21) and the hematopoietic cell-restricted 78-80 kDa TNF RII (22, 23), both of which are also expressed as homotrimers (1, 24). Both type I and type II receptors bind TNF-α with comparable affinity and can promote NF-κB activation (25-28). Only TNF RI, however, contains a cytoplasmic death domain which triggers the activation of apoptosis (3, 29). Soluble forms of both types of receptors are released into human serum and urine and can neutralize the biological activity of TNF-α (30-32).

II. OVERVIEW

A. PRINCIPLE OF THE ASSAY

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

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
Mean (pg/mL)	155	304	606	155	298	592
Standard Deviation	4.08	6.62	18.3	11.3	21.8	49.9
CV%	2.6	2.2	3.0	7.3	7.3	8.4

B. RECOVERY

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

Sample Type	Average % Recovery	Range %
Cell culture media (n=4)	103	98 - 110
Human serum (n=4)	88	79 - 98
EDTA plasma (n=4)	90	75 - 101
Heparin plasma (n=4)	90	82 - 95

C. SENSITIVITY

Twenty-five assays were evaluated and the minimum detectable dose (MDD) of human TNF-α ranged from 2.09-6.23 pg/mL. The mean MDD was 4.00 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 highly purified *E. coli*-expressed recombinant human TNF-α produced at R&D Systems.

The NIBSC/WHO 3rd International Standard for TNF-α (12/154), which was intended as a potency standard, was evaluated in this kit. The dose response curve of the International Standard (12/154) parallels the Valukine standard curve. To convert sample values obtained with the Valukine Human TNF-α kit to approximate NIBSC 12/154 units, use the equation below.

NIBSC (12/154) approximate value (IU/mL) = 0.072 × Valukine Human TNF-α value (pg/mL)

Note: Based on data generated in January 2018.

E. LINEARITY

To assess the linearity of the assay, samples were spiked with high concentrations of human TNF-α in various matrices and diluted with Calibrator Diluent RD6-12 to produce samples with values within the dynamic range of the assay.

Dilution		Cell culture supernates* (n=4)	Human serum (n=4)	Human EDTA plasma (n=4)	Human heparin plasma (n=4)
1:2	Average % of Expected	97	107	105	105
	Range (%)	93-100	103-109	103-108	103-108
1:4	Average % of Expected	98	112	108	108
	Range (%)	94-102	110-116	103-115	104-117
1:8	Average % of Expected	98	113	106	111
	Range (%)	93-107	110-118	102-113	105-119
1:16	Average % of Expected	99	119	110	116
	Range (%)	90-107	113-124	103-117	108-123

*Samples were diluted prior to assay.

F. SAMPLE VALUES

Human serum/plasma - Thirty serum and plasma samples from apparently healthy volunteers were evaluated for the presence of human TNF-α in this assay. All samples measured less than the lowest standard, 15.6 pg/mL. No medical histories were available for the donors used in this study.

Cell Culture Supernates - Human peripheral blood mononuclear cells (1×10^6 cells/mL) were cultured in RPMI1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were unstimulated or stimulated with 10 ng/mL of PMA and 500 ng/mL of Ionomycin Calcium Salt (Tocris, Catalog # 1704) for 24 hours. Aliquots of the culture supernates were removed, assayed for levels of human TNF-α, and measured 1355 pg/mL and 15,240 pg/mL, respectively.

$CD4^+$ T cells were isolated from human PBMCs using the MagCellect™ Human $CD4^+$ T cell Isolation Kit (R&D Systems, Catalog # MAGH102) before being differentiated into Th1 cells for 5 days and then treated with 10 ng/mL of PMA and 500 ng/mL of Ionomycin for an additional 24 hours. An aliquot of the cell culture supernate was removed, assayed for human TNF-α, and measured 81,100 pg/mL.

Human monocyte-derived macrophages (MDM) were obtained from PBMCs and attached monocytes were cultured in RPMI supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 100 ng/mL of recombinant human M-CSF for 5 days. Cells were treated with 1 µg/mL LPS and 40 ng/mL of recombinant human IFN-γ overnight. An aliquot of the cell culture supernate was removed, assayed for human TNF-α, and measured 8550 pg/mL.

G. SPECIFICITY

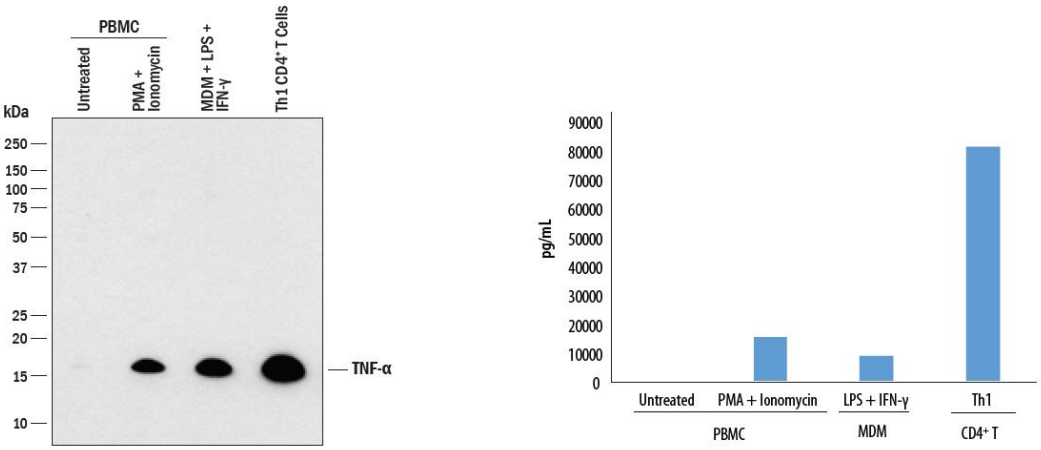
This assay recognizes both natural and recombinant human TNF-α.

The following factors were prepared at 50 ng/mL and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range rhTNF-α control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human		Other Recombinant	
CD40	TNF-β	Bovine TNF-α	Guinea Pig TNF-α
CD40 Ligand	TNF RII	Canine TNF-α	Mouse TNF-α
Fas Ligand	TRANCE	Cotton rat TNF-α	Porcine TNF-α
LIGHT	TRAIL	Equine TNF-α	rat TNF-α
TL1A		Feline TNF-α	rhesus macaque TNF-α

Recombinant human Pro-TNF-α does not interfere but cross-reacts approximately 1.5% in this assay.

Recombinant human TNF RI does not cross-react but interferes at concentrations > 2.0 ng/mL in this assay.

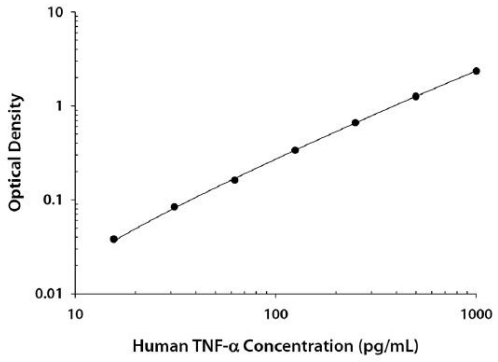


Conditioned media samples were analyzed by Western Blot and Valukine ELISA. Human PBMCs were left untreated, or treated with 10 ng/mL of PMA and 500 ng/mL Ionomycin calcium salt (Tocris, Catalog # 1704) for 24 hours. Human PBMC derived monocytes were differentiated with 100 ng/mL recombinant human M-CSF (R&D Systems, Catalog # 216-MC/CF) for 5-6 days into MDM cells and then treated with 1 μg/mL LPS and 40 ng/mL recombinant human IFN-γ (R&D Systems, Catalog # 285-IF/CF) for an additional 48 hours. $CD4^+$ T cells were isolated from human PBMCs using the MagCellect ™ Human $CD4^+$ T cell Isolation Kit (R&D Systems, Catalog # MAGH102) before being differentiated into Th1 cells for 5 days and then treated with 10 ng/mL of PMA and 500 ng/mL of Ionomycin for an additional 24 hours. For Western blot, all samples were initially diluted 1:2 before being resolved under reducing SDS-PAGE conditions, transferred to a PVDF membrane, and immunoblotted with Goat anti-Human TNF-α (R&D Systems, Catalog # AF-210-NA). The Western Blot shows a direct correlation with ELISA value for these samples.

IV. EXPERIMENT

EXAMPLE STANDARD

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



(pg/mL)	O.D.	Average	Corrected
0	0.054 0.056	0.055	—
15.6	0.092 0.093	0.093	0.038
31.3	0.138 0.139	0.139	0.084
62.5	0.214 0.220	0.217	0.162
125	0.390 0.394	0.392	0.337
250	0.710 0.726	0.718	0.663
500	1.300 1.326	1.313	1.258
1000	2.382 2.432	2.407	2.352

V. KIT COMPONENTS AND STORAGE

A. MATERIALS PROVIDED

Parts	Description	Size
Human TNF-α Microplate	96 well polystyrene microplate (12 strips of 8 wells) coated with an antibody against human TNF-α	1 plate
Human TNF-α Conjugate	Solution of an antibody against human TNF-α conjugated to horseradish peroxidase	1 vial
Human TNF-α Standard	Recombinant human TNF-α in a buffered protein base; lyophilized	2 vials
Assay Diluent RD1F	A buffered protein base with preservatives. *May contain a precipitate. Mix well before and during use.*	1 vial
Calibrator Diluent RD6-12	A buffered protein base used to dilute standard and 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	2 vials
Stop Solution	2 N sulfuric acid	1 vial
Plate Covers	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="7">Opened/ Reconstituted Reagents</td><td>Wash Buffer (1×)</td><td rowspan="6">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>Assay Diluent RD1F</td></tr>
<tr><td>Calibrator Diluent RD6-12</td></tr>
<tr><td>Standard</td><td>Use a new standard for each assay. Discard after use.</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch 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.
- Horizontal orbital microplate shaker capable of maintaining a speed of 500±50 rpm.

D. PRECAUTION

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

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 RD6-12.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 × g. Remove serum and assay immediately or aliquot and store samples at ≤ -20°C. 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 15 minutes at 1000 × g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Samples may require dilution with Calibrator Diluent RD6-12.

Note: *Citrate plasma was not validated for use in this assay.*

B. REAGENT PREPARATION

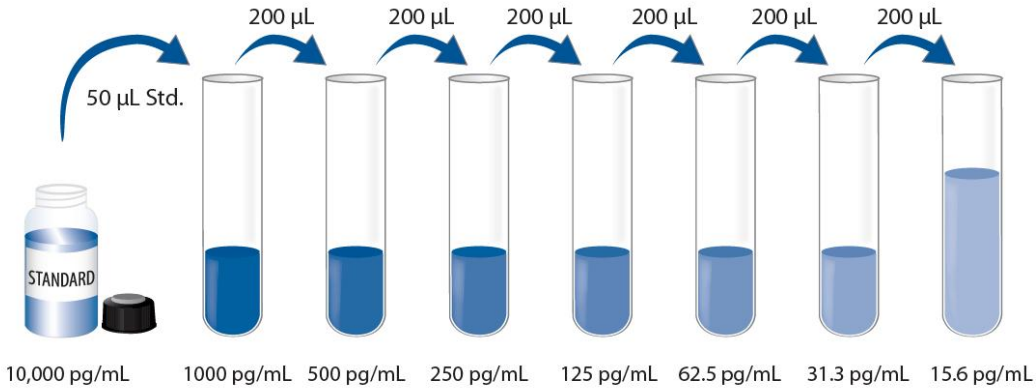
Note: Bring all reagents to room temperature before use.

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×).

TNF-α Standard - Refer to the vial label for reconstitution volume.* This reconstitution produces a stock solution of 10,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

*If you have any question, please seek help from our Technical Support.

Pipette 450 µL of Calibrator Diluent RD6-12 into the 1000 pg/mL tube. Pipette 200 µL into the other tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. The Calibrator Diluent RD6-12 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 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 RD1F to each well. *Assay Diluent RD1F may have a precipitate present. Mix well before and during use.*
4. Add 50 µL of Standard or sample per well. Cover with the adhesive strip provided. **Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500±50 rpm.** A plate layout is provided for a record of standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Solution (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 200 µL of human TNF-α Conjugate to each well. Cover with a new adhesive strip. **Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500±50 rpm. Avoid placing the plate in direct light.**
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of TMB Substrate Solution to each well. **Incubate for 30 minutes at room temperature. Avoid placing the plate in direct light.**
9. Add 50 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. 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 human 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.

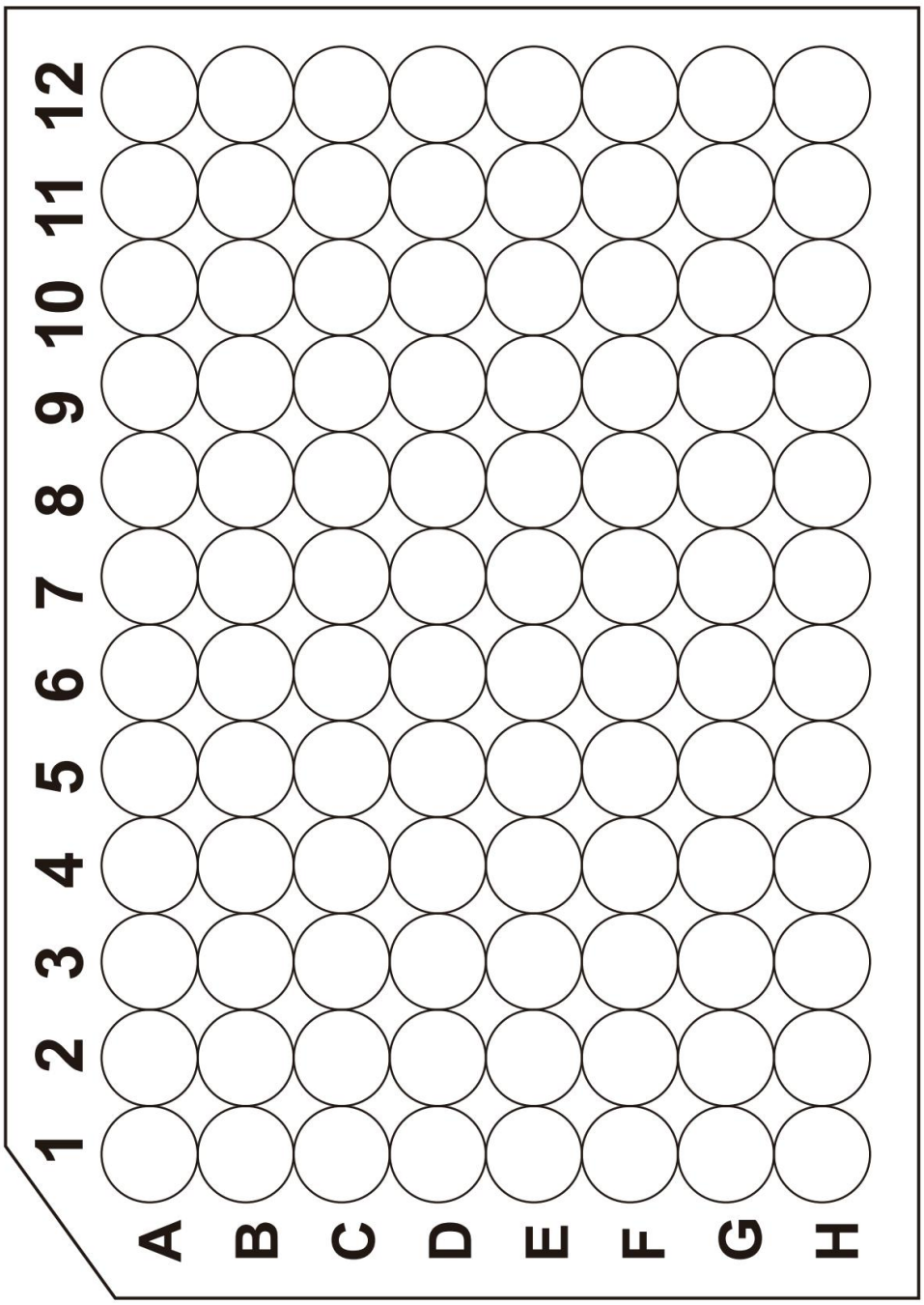
If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

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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

人 TNF-α Valukine™ ELISA 试剂盒

目录号：VAL105G

适用于定量检测天然和重组人肿瘤坏死因子-α（TNF-α）的含量

科研专用，不可用于临床诊断

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版本号 202605.6

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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、细胞凋亡和类脂代谢中起中心作用（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）。

人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177 aa的胞外域（ECD）组成（12, 13）。在ECD区，人TNF-α和猕猴有97%氨基酸序列同源性，与牛、犬、棉鼠、马、猫、小鼠、猪、大鼠有71-92%的氨基酸序列同源性。它可以由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在细胞内组装并在细胞表面表达形成非共价连接的同源三聚体（14）。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到信号（15, 16）。TACE/ADAM17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，它是由TNF-α胞外可溶性结构构成的三聚体，分子量为55 kDa（17-19）。

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（20, 21）；TNF RII分子量为78-80 kDa，仅限于造血细胞表达（22, 23）。两者都是以同源三聚体形式表达（1, 24）；TNF-α和TNF RI和TNF RII结合的亲和力相似，可促进NF-κB的激活（25-28）。然而，仅有TNF RI具有细胞死亡结构域，能引发细胞凋亡（3, 29）。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-α的活性（30-32）。

II. 概述

A. 检测原理

本实验采用双抗体夹心ELISA法。抗人TNF-α单抗包被于微孔板上，样品和标准品中的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人TNF-α抗体，与结合在微孔板上的TNF-α结合而形成免疫复合物，游离的成分被洗去；加入TMB底物溶液（显色剂），溶液颜色逐渐变成蓝色，加入终止液溶液变黄并且停止变化。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、人血清和人血浆样本；
- 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。

	板内精确度			板间精确度		
样本	1	2	3	1	2	3
平均值 (pg/mL)	155	304	606	155	298	592
标准差	4.08	6.62	18.3	11.3	21.8	49.9
CV%	2.6	2.2	3.0	7.3	7.3	8.4

B. 回收率

在不同类型样本中掺入检测范围内不同水平的人TNF-α，测定其回收率。

样本类型	平均回收率%	范围%
细胞培养上清 (n=4)	103	98 - 110
人血清 (n=4)	88	79 - 98
人EDTA血浆 (n=4)	90	75 - 101
人肝素血浆 (n=4)	90	82 - 95

C. 灵敏度

对25次检测进行了评估，人TNF-α的最低可测剂量（MDD）范围为2.09至6.23 pg/mL，平均最低可测剂量（MDD）为4.00 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试剂盒经由R&D Systems生产的大肠杆菌表达的高纯度重组人TNF-α蛋白所校正。

NIBSC/WHO TNF-α第3代国际标准品(12/154)作为效价标准，在本试剂盒中进行了评估。NIBSC/WHO标准品是大肠杆菌来源的重组人TNF-α。国际标准品(12/154)的剂量反应曲线与Valukine 标准曲线平行。若要将使用Valukine Human TNF-α kit获得的样本值转换为NIBSC 12/154的近似单位，请使用以下公式：

NIBSC (12/154) approximate value (IU/mL) = 0.072 × Valukine Human TNF-α value (pg/mL)

注：基于2018年1月产生的数据。

E. 线性

在不同类型样本中掺入高浓度的人TNF-α，然后用标准品稀释液RD6-12将样本稀释到检测范围内，测定其线性。

稀释倍数		细胞培养上清* (n=4)	人血清 (n=4)	人EDTA血浆 (n=4)	人肝素血浆 (n=4)
1:2	平均值/期待值 (%)	97	107	105	105
	范围 (%)	93-100	103-109	103-108	103-108
1:4	平均值/期待值 (%)	98	112	108	108
	范围 (%)	94-102	110-116	103-115	104-117
1:8	平均值/期待值 (%)	98	113	106	111
	范围 (%)	93-107	110-118	102-113	105-119
1:16	平均值/期待值 (%)	99	119	110	116
	范围 (%)	90-107	113-124	103-117	108-123

*样本在检测前已进行稀释。

F. 样本预值

人血清/血浆 - 采用本检测方法对来自表观健康志愿者的30份血清和血浆样本进行了人TNF-α检测。所有样本的检测结果均低于最低标准品浓度，即15.6 pg/mL。本研究未获得所用样本捐献者的既往病史信息。

细胞培养上清液 - 将人外周血单个核细胞（1×10⁶个细胞/mL）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RPMI1640培养基中。细胞未经刺激，或用10 ng/mL PMA和500 ng/mL离子霉素钙盐刺激24小时。取培养上清液的等分试样，检测人TNF-α水平，未刺激组和刺激组的检测值分别为1355 pg/mL和15,240 pg/mL。

使用MagCellect™人CD4⁺ T细胞分离试剂盒（R&D Systems，货号：MAGH102）从人PBMC中分离出CD4⁺ T细胞，随后分化为Th1细胞5天，再用10 ng/mL PMA和500 ng/mL离子霉素处理24小时。取细胞培养上清液的等分试样，检测人TNF-α，检测值为81,100 pg/mL。

人单核细胞来源的巨噬细胞从PBMC中获得，将贴壁的单核细胞在添加了10%胎牛血清、100 U/mL青霉素、100 µg/mL硫酸链霉素和100 ng/mL重组人M-CSF的RPMI培养基中培养5天。细胞用1 µg/mL LPS和40 ng/mL重组人IFN-γ处理过夜。取细胞培养上清液的等分试样，检测人TNF-α，检测值为8,550 pg/mL。

G. 特异性

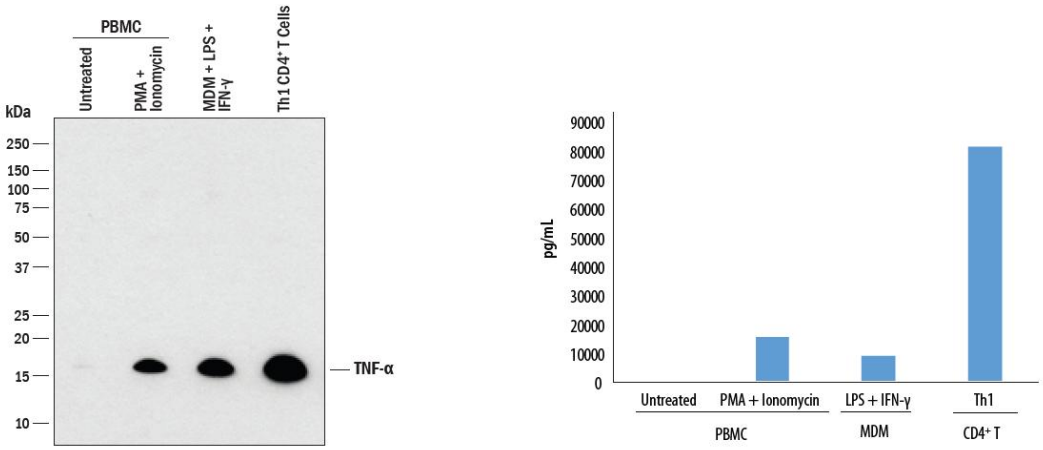
此ELISA法可检测天然及重组人TNF-α蛋白。

将以下因子配制成50 ng/mL的浓度来检测与人TNF-α的交叉反应。将50 ng/mL的干扰因子掺入中间范围的重组人TNF-α对照品中，来检测对人TNF-α的干扰。没有观察到明显的交叉反应或干扰。

重组人蛋白		**其他重组蛋白**	
CD40	TNF-β	牛 TNF-α	豚鼠 TNF-α
CD40 Ligand	TNF RII	犬 TNF-α	小鼠 TNF-α
Fas Ligand	TRANCE	棉鼠 TNF-α	猪 TNF-α
LIGHT	TRAIL	马 TNF-α	大鼠 TNF-α
TL1A		猫 TNF-α	恒河猕猴 TNF-α

重组人Pro-TNF-α 并不干扰实验结果，但是测试中观察到约1.5%的交叉反应。

重组人TNF RI在本实验中不发生交叉反应，但浓度为> 2.0 ng/mL时会对实验产生干扰。

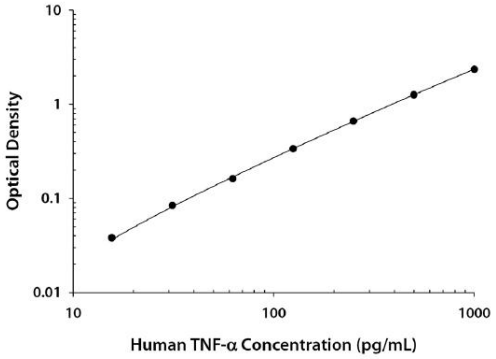


采用Western Blot和 Valukine ELISA对条件培养基样本进行了分析。人PBMC未经处理，或用10 ng/mL PMA和500 ng/mL离子霉素钙盐（Tocris，货号：1704）处理24小时。人PBMC来源的单核细胞，用100 ng/mL重组人M-CSF（R&D Systems，货号：216-MC/CF）分化5-6天成为MDM细胞，然后用1 µg/mL LPS和40 ng/mL重组人IFN-γ（R&D Systems，货号：285-IF/CF）再处理4小时。使用 MagCellect™人 $CD4^+$ T细胞分离试剂盒（R&D Systems，货号：MAGH102）从人PBMC中分离 $CD4^+$ T细胞，然后分化为Th1细胞5天，再用10 ng/mL PMA和500 ng/mL离子霉素处理24小时。对于Western Blot，所有样本初始稀释2倍，然后在还原性SDS-PAGE条件下分离，转移至PVDF膜，并用山羊抗人TNF-α（R&D Systems，货号：AF-210-NA）进行免疫印迹。Western Blot 结果显示，对于这些样本，其信号强度与ELISA检测值呈直接相关性。

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。



(pg/mL)	O.D.	Average	Corrected
0	0.054 0.056	0.055	—
15.6	0.092 0.093	0.093	0.038
31.3	0.138 0.139	0.139	0.084
62.5	0.214 0.220	0.217	0.162
125	0.390 0.394	0.392	0.337
250	0.710 0.726	0.718	0.663
500	1.300 1.326	1.313	1.258
1000	2.382 2.432	2.407	2.352

V. 试剂盒组成及储存

A. 试剂盒组成

组成	描述	规格
Human TNF-α Microplate	包被抗体的 96 孔聚苯乙烯板，8 孔×12 条	1 块板
Human TNF-α Conjugate	酶标检测人 TNF-α抗体	1 瓶
Human TNF-α Standard	标准品（冻干），参考瓶身标签进行重溶	2 瓶
Assay Diluent RD1F	检测液 RD1F。*可能有沉淀，用前混匀。*	1 瓶
Calibrator Diluent RD6-12	标准品稀释液 RD6-12，用于稀释标准品和样本	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	2 瓶
Stop Solution	终止液	1 瓶
Plate Covers	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="6">2-8℃储存，最多 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>检测液 RD1F</td></tr>
<tr><td>标准品稀释液 RD6-12</td></tr>
<tr><td>标准品</td><td>每次检测使用新配制的标准品。使用后丢弃</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，最多 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
- 振荡器（速度可调至 500±50 rpm）

D. 注意事项

本试剂盒提供的终止液为酸性溶液。

本试剂盒中的某些组分含有防腐剂，可能引起过敏性皮肤反应。避免吸入雾状液滴。

穿戴防护手套、防护服、眼罩和面罩。操作后彻底清洗双手。

VI. 实验前准备

A. 样品收集及储存

以下列出的样本采集和保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RD6-12稀释。

血清样本：用血清分离管(SST)分离血清。使血样室温凝集30分钟，然后1000 × g离心15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。

血浆样本：使用EDTA或肝素作为抗凝剂收集血浆。然后1000 × g离心15分钟，需在30分钟内收集血浆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。

注意：*本检测方法尚未对枸橼酸盐血浆进行验证。*

B. 检测前准备工作

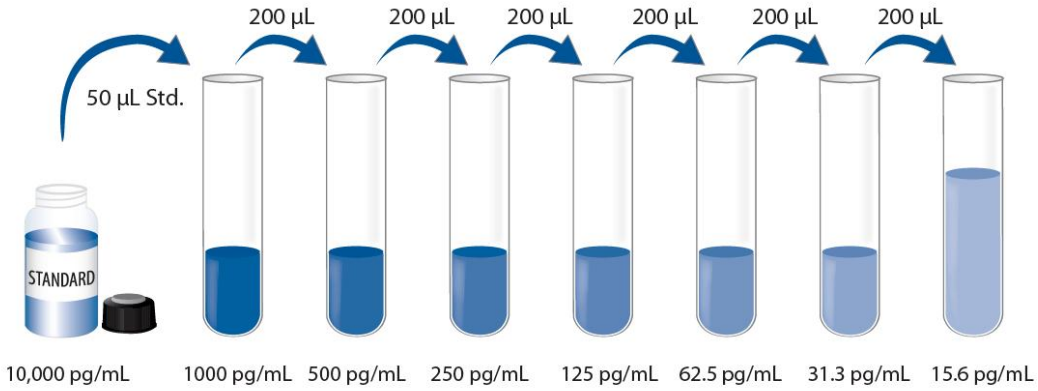
使用前请将所有试剂放置于室温

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浓缩洗涤液用蒸馏水或去离子水稀释配制成500 mL工作浓度的洗涤液（1×）。

标准品：依照标准品瓶标签上注明的体积重溶冻干标准品*，得到浓度为10,000 pg/mL标准品母液。轻轻震摇至少15分钟，其充分溶解。

*如有疑问，请咨询我们的技术支持。

在1000 pg/mL的稀释管中加入450 µL标准品稀释液RD6-12，其余每个稀释管中加入200 µL标准品稀释液RD6-12。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。1000 pg/mL的标准品可用作标准曲线最高点，标准品稀释液RD6-12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. 向每个孔中加入50 μL的检测液RD1F。*检测液RD1F中可能会有沉淀。使用前及使用过程中请充分混匀。*
4. 分别将标准品或者样本加入相应孔中，每孔50 μL。用封板膜封住反应孔，**室温500±50 rpm水平振荡孵育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3次，共洗4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200 μL酶标检测抗体。用封板膜封住反应孔，**室温500±50 rpm水平振荡孵育2小时。注意避光**；
7. 重复第5步洗板操作；
8. 在每个微孔内加入200 μL TMB底物溶液，**室温500±50 rpm水平振荡孵育30分钟。注意避光**；
9. 在每个微孔内加入50 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。数据可以通过绘制人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。

VIII. 参考文献

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96 孔模板图

请使用 96 孔模板图来记录标准品及样本在板内的位置

