PRODUCT INFORMATION & MANUAL

Human TNF-α Valukine™ ELISA

VAL105

For the detection of human TNF-α

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

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Novus kits are guaranteed for 3 months from date of receipt

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I. BACKGROUND

Tumor necrosis factor alpha (TNF-α), also known as cachectin, 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 extracllular 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 55-66 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 NFκB 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. A monoclonal antibody specific for TNF-α has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and an TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of 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 INDIAGNOSTIC PROCEDURES.**
- 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 Diluent 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)	81.6	264	569	83.1	278	587
Standard Deviation	4.81	15.8	32.5	5.96	25.4	54.5
CV%	5.9	6.0	5.7	7.2	9.1	9.3

B. RECOVERY

The recovery of TNF-α spiked to different levels in cell culture media was evaluated. The recovery ranged from 84-101% with an average of 89%.

C. SENSITIVITY

The minimum detectable dose (MDD) of TNF-α is typically less than 7.8 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.

E. LINEARITY

To assess the linearity of the assay, four cell culture media samples were spiked with high concentrations of TNF-α and diluted with Diluent 1× to produce samples with values within the dynamic range of the assay.

Dilution	Average % of Expected	Range (%)
1:2	106	99 – 110
1:4	102	86 – 116
1:8	103	83 – 117
1:16	100	80 – 120

F. SAMPLE VALUES

Human peripheral blood mononuclear cells (1×10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate and stimulated for 6 days with 10 µg/mL PHA. An aliquot of the cell culture supernate was removed, assayed for levels of natural TNF-α and measured 1728 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.

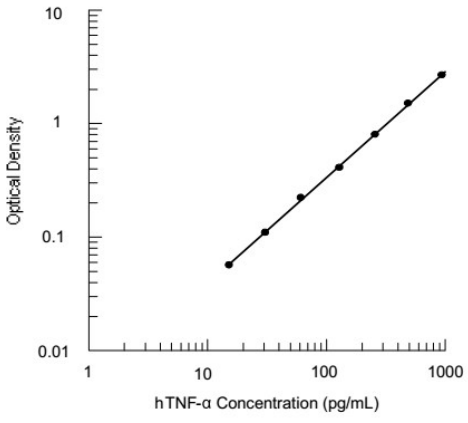
Recombinant human:	**Recombinant mouse:**	**Recombinant equine:**
IL-1α	IL-1α	TNF-α
IL-1β	IL-1β	
IL-1ra	IL-3	**Recombinant porcine:**
IL-2	IL-4	TNF-α
IL-3	IL-5	
IL-4	IL-6	**Recombinant rhesus macaque:**
IL-5	IL-7	TNF-α
IL-6	IL-9	
IL-8		
IL-10		
IL-11		
TNF-β		
sTNF RI		
sTNF RII		

Less than 1% cross-reactivity was observed with recombinant canine, mouse and rat TNF-α

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	OD	Average	Corrected
0	0.053 0.053	0.053	——
15.6	0.107 0.109	0.108	0.055
31.2	0.156 0.164	0.16	0.107
62.5	0.279 0.275	0.277	0.224
125	0.449 0.437	0.443	0.39
250	0.765 0.82	0.793	0.74
500	1.468 1.455	1.462	1.409
1000	2.628 2.79	2.709	2.656

V. KIT COMPONENTS AND STORAGE

A. MATERIALS PROVIDED

Parts	Description	Size
TNF-α Microplate	96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against human TNF-α	1 plate
TNF-α Conjugate	21 mL/vial of polyclonal antibody against TNF-α conjugated to horseradish peroxidase with preservatives	1 vial
TNF-α Standard	Recombinant human TNF-α in a buffered protein base with preservatives; lyophilized	1 vial
Calibrator Diluent RD5P（5×）	21 mL/vial of a 5× concentrated buffered protein base with preservatives	1 vial
Wash Buffer Concentrate（25×）	21 mL/vial of a 25× concentrated solution of buffered surfactant with preservatives	1 vial
Color Reagent A	12.5 mL/vial of stabilized hydrogen peroxide	1 vial
Color Reagent B	12.5 mL/vial of stabilized chromogen (tetramethylbenzidine)	1 vial
Stop Solution 1	6 mL/vial of 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℃. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="8">Opened/ Reconstituted Reagents</td><td>Diluted Wash Solution</td><td rowspan="6">May be stored for up to 1 month at 2-8℃.*</td></tr>
<tr><td>Stop Solution 1</td></tr>
<tr><td>Dilution 1×</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>Unmixed Substrate A</td></tr>
<tr><td>Unmixed Substrate B</td></tr>
<tr><td>Standard</td><td>Aliquot and store for up to 1 month at <-20 °C in a manual defrost freezer.* Avoid repeated freezed-thaw cycles.</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℃.*</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

The Stop solution 1 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

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤-20℃. Avoid repeated freeze-thaw cycles. Samples may require dilution with Diluent 1×.

B. REAGENT PREPARATION

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

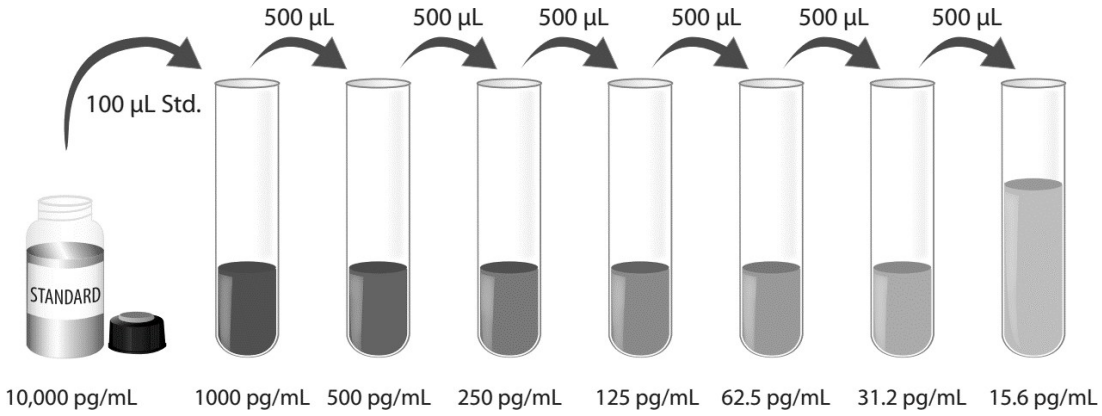
Wash Solution - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Solution Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Substrates A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Diluent 1× - Add 20 mL of Diluent Concentrate 5× into 80 mL of deionized or distilled water to prepare 100 mL of Diluent 1×.

TNF-α Standard-Refer to the vial label for reconstitution volume. Reconstitute the TNF-α Standard with deionized or distilled water. 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.

Pipette 900 µL of Diluent 1× into the 1000 pg/mL tube. Pipette 500 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 Diluent 1× 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.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop solution 1 should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop solution 1. Wells that are green in color indicate that the Stop solution 1 has not mixed thoroughly with the Substrate Solution.

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 100 µL of Standard, sample, or control per well. 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.
4. 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.

5. Add 200 µL of TNF-α Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
6. Repeat the aspiration/wash as in step 4.
7. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light**.
8. Add 50 µL of Stop Solution 1 to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well within 30 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.
10. **CALCULATION OF RESULTS：** Average the duplicate readings for each standard, control, 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 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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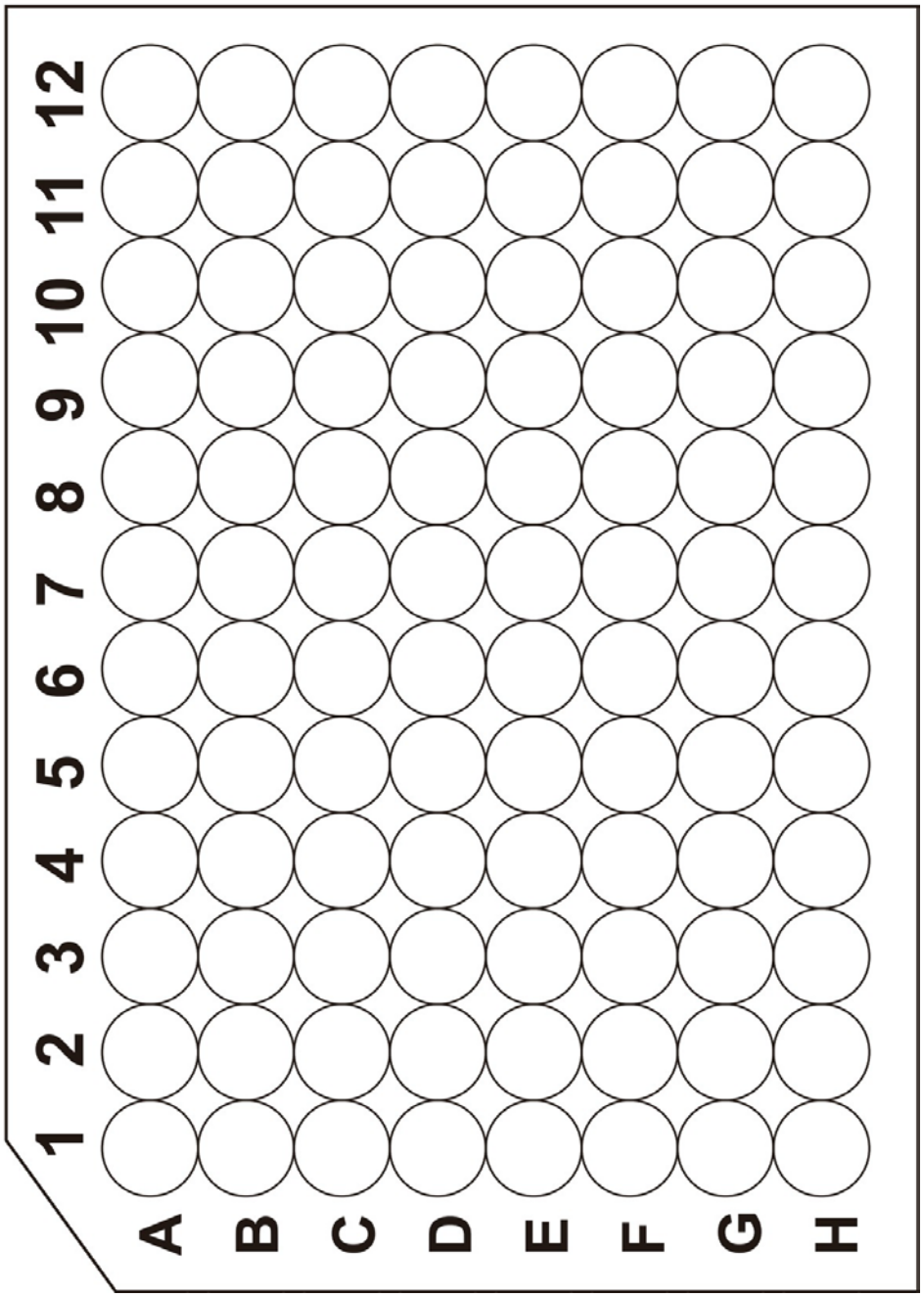
IX. TROUBLESHOOTING

Problem	Probable Cause	Suggestion
No signal	Failure to add all components	Prepare a check-list and add the components in the correct order
Low signal	Not enough supernatant per well	Check the protein concentration. Add more supernatant.
High background	Wells are not washed enough.	Wash plates thoroughly after incubation with detecting.

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：VAL105

适用于定量测定细胞培养上清液中人肿瘤坏死因子-α（TNF-α）的含量

科研专用，不可用于临床诊断

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Novus 试剂盒确保在你收货日期 3 个月内有效

目录

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到一个中心作用（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）。

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（20,21）和TNF RII 结合的亲和力相似，可促进NFκB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多抗，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；加入底物溶液（显色剂），溶液颜色逐渐变成蓝色，加入终止液1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；**
- 试剂盒请在有效期内使用；
- 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，应将样本用稀释剂（1×）稀释后重新检测；若细胞培养上清液样本需分布稀释，除最后一步用稀释剂稀释外，其它中间稀释可采用细胞培养基；
- 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、试剂盒的储存等。

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，以确定板内精确度。

	板内精确度			板间精确度		
样本	1	2	3	1	2	3
平均值 (pg/mL)	81.6	264	569	83.1	278	587
标准差	4.81	15.8	32.5	5.96	25.4	54.5
CV%	5.9	6.0	5.7	7.2	9.1	9.3

B. 回收率

在细胞培养基样本中掺入不同水平的人TNF-α，测定其回收率。回收率范围在84-101%，平均回收率在89%。

C. 灵敏度

人TNF-α的最低可测值一般小于7.8 pg/mL。

最低可测值是根据20个标准曲线零点吸光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重组人TNF-α蛋白所校正。

E. 线性

4个不同的样本中掺入高浓度的人TNF-α，然后用稀释剂（1×）将样本稀释到检测范围内，测定其线性。

稀释倍数	平均值/期待值（%）	范围 (%)
1:2	106	99 – 110
1:4	102	86 – 116
1:8	103	83 – 117
1:16	100	80 – 120

F. 样本预值

人的外周血单核细胞（1×10^6 细胞/mL）培养于含有10%胎牛血清的RPMI1640 培养基中， 细胞培养基还含有2 mM L-谷氨酰胺、50 µM β-巯基乙醇、100 U/mL 青霉素, 100 µg/mL 链霉素，另加10 µg/mL PHA 刺激细胞，培养6天。取细胞培养上清液测定TNF-α含量，结果为 1728 pg/mL。

G. 特异性

此 ELISA 法可检测天然及重组人 TNF-α蛋白。将以下因子用稀释剂（1×）配置成 50 ng/mL 的浓度来检测与人 TNF-α的交叉反应。将干扰因子掺入中间浓度重组人 TNF-α标准中，使其浓度到达 50 ng/mL，以此来检测对人 TNF-α的干扰。没有观察到明显的交叉反应或干扰。

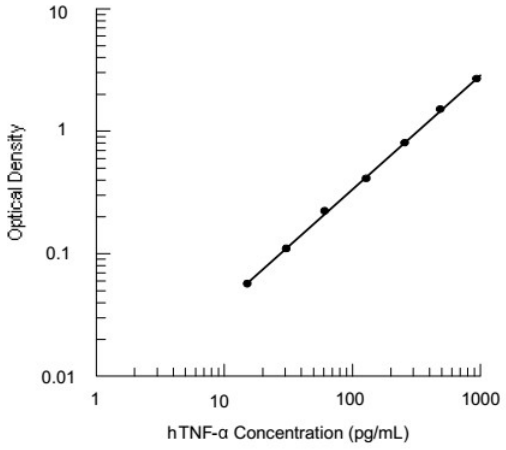
重组人蛋白:	重组小鼠蛋白:	重组马蛋白:
IL-1α	IL-1α	TNF-α
IL-1β	IL-1β	
IL-1ra	IL-3	**重组猪蛋白:**
IL-2	IL-4	TNF-α
IL-3	IL-5	
IL-4	IL-6	**重组恒河猴蛋白:**
IL-5	IL-7	TNF-α
IL-6	IL-9	
IL-8		
IL-10		
IL-11		
TNF-β		
sTNF RI		
sTNF RII		

与犬科，小鼠和大鼠的 TNF-α的特异性实验中，观察到少于1%的交叉反应。

IV. 实验

实验标准

提供的标准曲线数据仅供参考，应根据同次试验所绘标准曲线计算样本含量。



pg/mL	OD	Average	Corrected
0	0.053 0.053	0.053	——
15.6	0.107 0.109	0.108	0.055
31.2	0.156 0.164	0.16	0.107
62.5	0.279 0.275	0.277	0.224
125	0.449 0.437	0.443	0.39
250	0.765 0.82	0.793	0.74
500	1.468 1.455	1.462	1.409
1000	2.628 2.79	2.709	2.656

V. 试剂盒组成及储存

A. 试剂盒组成

组成	描述	规格
TNF-α Microplate	包被抗体的96孔聚苯乙烯板，8孔×12 条	1 块板
TNF-α Conjugate	酶标检测TNF-α抗体，21 mL/瓶	1 瓶
TNF-α Standard	标准品（冻干）	1 瓶
Calibrator Diluent RD5P（5×）	浓缩标准品稀释缓冲液（5×），21 mL/瓶	1 瓶
Wash Buffer Concentrate（25×）	浓缩洗涤缓冲液（25×），21 mL/瓶	1 瓶
Color Reagent A	显色液 A，12.5 mL/瓶	1 瓶
Color Reagent B	显色液 B，12.5 mL/瓶	1 瓶
Stop Solution 1	终止液 1，6 mL瓶	1 瓶
Plate Covers	封板胶纸	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>洗涤缓冲液（1×）</td><td rowspan="6">2-8 ℃储存，30 天*</td></tr>
<tr><td>终止液 1</td></tr>
<tr><td>稀释液（1×）</td></tr>
<tr><td>酶标检测抗体</td></tr>
<tr><td>显色剂 A</td></tr>
<tr><td>显色剂 B</td></tr>
<tr><td>标准品</td><td>分装，-20℃以下冰箱储存 30 天*；避免反复冻融。</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

- 试剂盒中的终止液 1 是酸性溶液，使用时请做好眼镜、手、面部及衣服的防护。

VI. 实验前准备

A. 样品收集及储存

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℃电冰箱内，避免反复冻融。样本可能需要用稀释剂（1×）稀释。

B. 检测前准备工作

使用前请将所有试剂放置于室温.

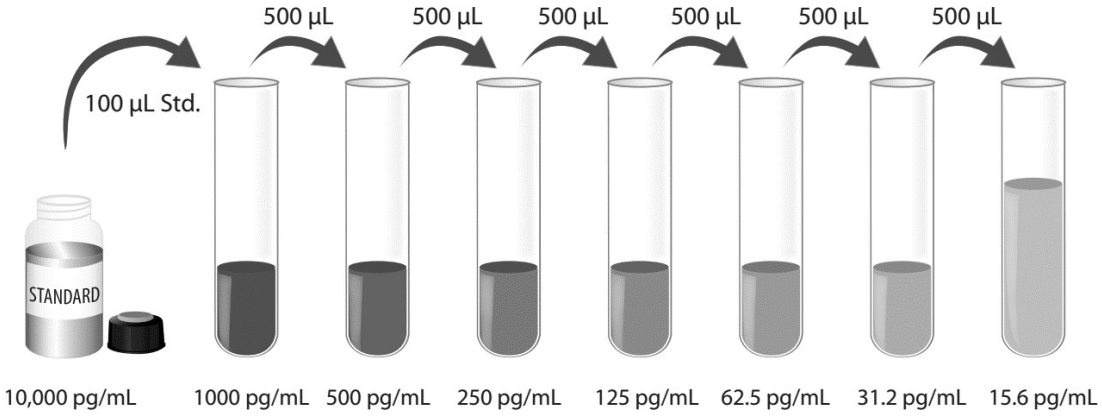
洗涤液：从冰箱中取出的浓缩洗涤液可能有结晶，属于正常现象；放置室温，轻摇混匀，待结晶完成溶解后再配制洗涤液。可将20 mL 浓缩洗涤液用蒸馏水或去离子水稀释配置成500mL 工作浓缩的洗涤液。未用完的放回4℃。

显色剂：按当次试验所需要用量将显色剂A和显色剂B 等体积混合，避光；在使用前15 分钟准备，仅供当日使用；每孔需100 µL。

稀释剂（1×）：可将20 mL 浓缩稀释剂用80 mL 蒸馏水或去离子水稀释配置成100 mL工作浓度的稀释剂。

标准品：依照标准品标签上注明的重溶体积，用稀释剂（1×）重溶冻干标准品，得到浓度为1000 pg/mL 标准品母液。轻轻震摇至少 15 分钟，其充分溶解。

在1000 pg/mL 的稀释管中加入900 µL 稀释剂（1×），其余每个稀释管中加入 500 µL 稀释剂（1×）。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，稀释剂（1×）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，配置不同浓度标准品、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，正确使用封板胶纸可保证结果的准确性；
- 混合后的显色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1上板顺序应同显色底物上板顺序一致；加入终止液1后，孔内颜色由蓝变黄；若空内有绿色，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. 分别将不同浓度标准品，实验样本或者质控品加入相应孔中，每孔100 µL。用封板胶纸封住反应孔，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；
4. 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。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；
5. 在每个微孔内加入200 µL酶标检测抗体。用封板胶纸封住反应孔，室温孵育2小时；
6. 重复第4步洗板操作；
7. 在每个微孔内加入200 µL 显色底物，室温孵育30 分钟。**注意避光**；
8. 在每个微孔内加入50 µL 终止液1，孔内溶液颜色会从蓝色变为黄色。如果溶液颜色变为绿色或者颜色变化不一致，请轻拍微孔板，使溶液混合均匀；
9. 加入终止液1后30 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。如果没有使用双波长校正，结果准确度可能会受影响；
10. **计算结果：**每一个标准品及样本的复孔校正吸收光值（OD450-OD540/OD570）取平均值，再减去标准曲线零点的OD 值。利用酶标仪携带的软件，绘制一个 4 参数（4-PL）线性标准曲线，曲线横坐标为标准曲线点的人TNF-α浓度值，纵坐标为标准曲线点的OD平均值。通过样本的OD值，可从标准曲线上得到样本中人TNF-α的浓度。若样本经过稀释，计算浓度时应乘以稀释倍数。

VIII. 参考文献

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IX. 疑难解答

问题	可能的原因	解决方案
无信号	并未加入所有组分	准备一个检查表并按照正确的顺序添加组件
低信号	每孔内上清不够	检查待测蛋白浓度，可以添加更多的样本
高信号	洗涤不够充分	充分洗板，增加洗涤次数

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