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.

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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 202409.5

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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 55-66 kDa 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 neutralized the biological activity of TNF-α (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.
- 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 (1×) 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)

Two 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	1	2	3
Mean (pg/mL)	54.6	116	405	45.0	111
Standard Deviation	1.5	4.5	12.5	4.27	7.3
CV%	2.7	3.9	3.1	9.5	6.6

B. RECOVERY

The recovery of mouse TNF-α spiked to different levels throughout the range of the assay in cell culture media was evaluated. The mouse sample recovery ranged from 88 to 107% with an average of 94%.

The recovery of mouse TNF-α spiked to different levels throughout the range of the assay in mouse serum was evaluated. The recovery ranged from 75.3 to 89.3% with an average of 81.1%.

C. SENSITIVITY

The minimum detectable dose (MDD) of mouse TNF-α is typically less than 5.5 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 mouse TNF-α produced at R&D Systems.

E. LINEARITY

To assess the linearity of the assay, different samples were containing or spiked with high concentrations of mouse TNF-α and diluted with Calibrator Diluent (1×) to produce samples with values within the dynamic range of the assay.

Dilution	Average % of Expected	Range (%)
1:2	97	94-103
1:4	105	99-111
1:8	106	97-114
1:16	107	104-111

F. SAMPLE VALUES

Cell Culture Supernates - Mouse thymoma cells (EL-4; 2×10^5 cells/mL) were cultured for 4 days in DMEM supplemented with 10% fetal calf serum and stimulated with 10 µg/mL LPS and 100 ng/mL recombinant mouse IL-10. An aliquot of the cell culture supernate was removed, assayed for levels of natural mouse TNF-α and measured 1220 pg/ml.

Mouse serum - Four serum samples were evaluated for the presence of TNF-α in this assay. All samples measured ranged from 12.0 to 18.4 pg/mL with an average of 14.4 pg/mL.

G. SPECIFICITY

This assay recognizes both natural and recombinant mouse TNF-α. The following factors were prepared at 100 ng/mL and assayed for cross-reactivity. Preparations of the following factors at 100 ng/mL in a mid-range rmTNF-α control were assayed for interference. No significant cross-reactivity or interference was observed.

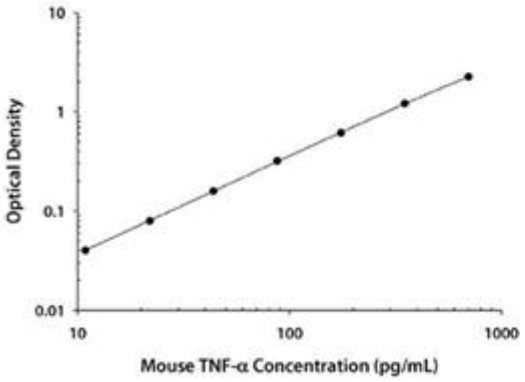
Recombinant mouse	Other Recombinant
CD40	Canine TNF-α
CD40 ligand	Human TNF-α
Fas	Porcine TNF-α
Fas ligand	
LIF	
OPG	
RANK	
RANK ligand	
TRAIL	
TROY	
TNF-β	

40% cross-reactivity was observed with recombinant rat TNF-α. Recombinant mouse TNF RI and recombinant mouse 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 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	0.020 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

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; lyophilized. Refer to the vial label for reconstitution volume	2 vials
Calibrator Diluent (5×)/RD5P	A 5× concentrated 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	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="6">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 fresh standard for each assay.
Standards may be stored for up to 1 month at -20 °C.*</td></tr>
<tr><td>Calibrator Diluent (5×)/RD5P</td><td>May be stored for up to 1 month at 2-8 °C.*
Use and discard diluted Calibrator Diluent (1×). Prepare fresh for each assay.</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the oil 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

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 (1×).

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 x 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 (1×).

B. SAMPLE PREPARATION

Mouse serum samples recommend a 2-fold dilution. A suggested 2-fold dilution is 100 µL of sample + 100 µL of Calibrator Diluent (1×). Optimal dilutions should be determined by the end user.

C. 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×).

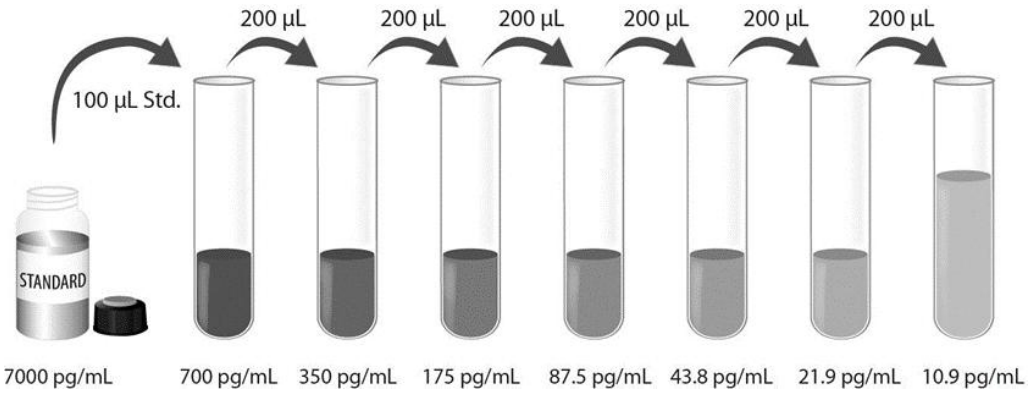
Calibrator Diluent (1×) - Use deionized or distilled water to prepare Calibrator Diluent (1×).

Mouse TNF-α Standard – Refer to the vial label for the reconstitution volume*. This reconstitution produces a stock solution of 7000 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 contact our Technical Support.

Pipette 900 µL of Calibrator Diluent (1×) into the 700 pg/mL tube. Pipette 200 µL of Calibrator Diluent (1×) 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 Calibrator Diluent (1×) serves as the zero

standard (0 pg/mL).



D. 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 Calibrator Diluent (1×) to each well.
4. Add 50 µL of Standard and sample 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.
5. Aspirate each well and wash, repeating the process three times for a total of four 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.

VIII. REFERENCES

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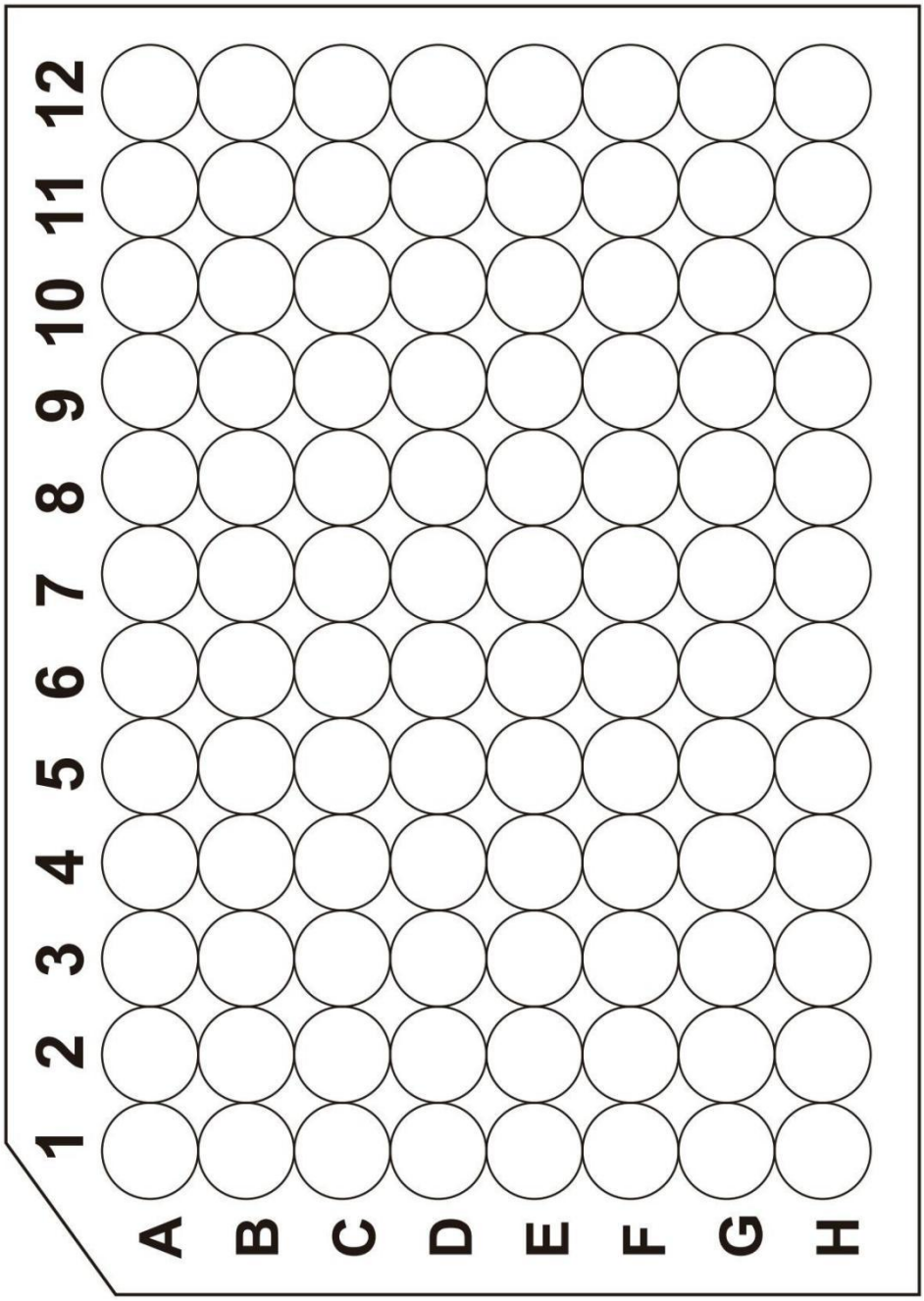
PLATE LAYOUT

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

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版本号 202409.5

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-α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（1×）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）

已知浓度的两个样本，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	1	2	3
平均值(pg/mL)	54.6	116	405	45.0	111
标准差	1.5	4.5	12.5	4.27	7.3
CV%	2.7	3.9	3.1	9.5	6.6

B. 回收率

在细胞培养基样本中掺入检测范围内不同水平的小鼠 TNF-α，测定其回收率。小鼠样本回收率范围在 88-107%，平均回收率在 94%。

在小鼠血清样本中掺入检测范围内不同水平的小鼠 TNF-α，测定其回收率。回收率范围在 75.3-89.3%，平均回收率在 81.1%。

C. 灵敏度

小鼠 TNF-α的最低可测剂量（MDD）一般小于 5.5 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 生产的大肠杆菌表达的高纯度重组小鼠 TNF-α蛋白所校正。

E. 线性

不同的样本中含有或掺入高浓度的小鼠 TNF-α，然后用标准品稀释液（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	97	94-103
1:4	105	99-111
1:8	106	97-114
1:16	107	104-111

F. 样本预值

细胞培养上清液 - 取小鼠胸腺瘤细胞（EL-4, 2×10^5 细胞/mL）培养于含有 10%胎牛血清的 DMEM 培养基中，另加 10 μg/mL LPS 和 100 ng/mL 重组小鼠 IL-10 刺激细胞，培养 4 天。取细胞培养上清液测定天然小鼠 TNF-α含量，结果为 1220 pg/mL。

小鼠血清样本 - 使用本试剂盒检测了 4 份小鼠血清样本中 TNF-α的水平。所有样本的检测值在 12.0-18.4 pg/mL 之间，平均值为 14.4 pg/mL。

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。

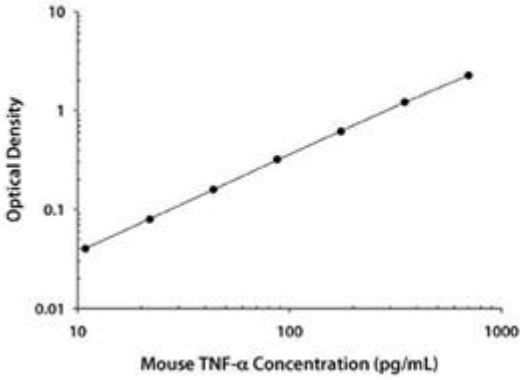
Recombinant mouse	**Other Recombinant**
CD40	Canine TNF-α
CD40 ligand	Human TNF-α
Fas	Porcine TNF-α
Fas ligand	
LIF	
OPG	
RANK	
RANK ligand	
TRAIL	
TROY	
TNF-β	

与重组大鼠TNF-α有40%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。



(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

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），参考瓶身标签进行重溶	2 瓶
Calibrator Diluent (5×)/RD5P	浓缩标准品稀释液（5×）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	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="6">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；
标准品-20℃储存，最多30天*</td></tr>
<tr><td>标准品稀释液（5×）/RD5P</td><td>2-8℃储存，最多 30 天*
请每次使用新鲜配制的1×标准品稀释液，多余的丢弃</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，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

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（1×）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 x 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（1×）稀释。

B. 样本准备工作

小鼠血清样本建议用标准品稀释液（1×）2倍稀释后进行检测，即100 µL血清+100 µL标准品稀释液（1×）。最佳稀释度应由最终用户确定。

C. 检测前准备工作

使用前请将所有试剂放置于室温。

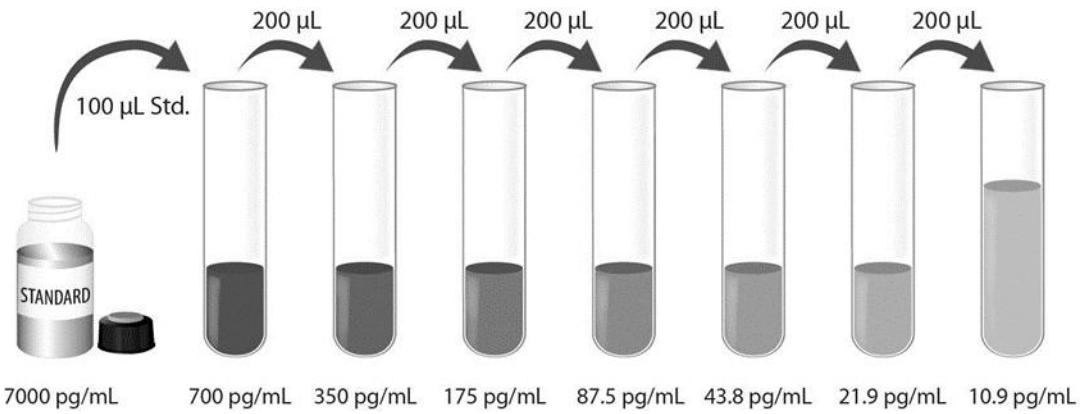
洗涤液（1×）：从冰箱中取出的浓缩洗涤液可能有结晶，属于正常现象；放置室温，轻摇混匀， 待结晶完全溶解后再配制洗涤液。可将20 mL浓缩洗涤液（25×）用蒸馏水或去离子水稀释配制成 500mL工作浓度的洗涤液（1×）。

标准品稀释液（1×）：使用去离子水或蒸馏水稀释配制成标准品稀释液（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。得到浓度为7000 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700 pg/mL的稀释管中加入900 µL标准品稀释液（1×），其余每个稀释管中加入200 µL标准品稀释液（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。700 pg/mL管作标准曲线最高点，标准品稀释液（1×）可用作标准曲线零点（0 pg/mL）。



D. 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标准品稀释液（1×）；
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。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。

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