Speedy™ Mouse TNF-alpha One-Step ELISA Kit Datasheet

Please read it entirely before use

Catalogue Number: SE60082

Size: 96T

Sensitivity: 0.2 pg/mL

Range: 1.95-125 pg/mL

Usage: For the quantitative detection of mouse TNF-alpha concentrations in serum, plasma and cell culture supernatant.

This product is for research use only and not for use in human or animal therapeutic or diagnostic.

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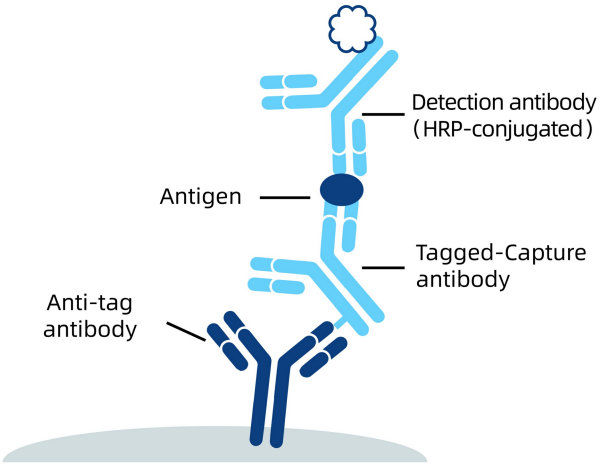
1. Background

TNF, also known as TNF-alpha, or cachectin, is a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. It is expressed as a 26 kDa membrane bound protein and is then cleaved by TNF-alpha converting enzyme (TACE) to release the soluble 17 kDa monomer, which forms homotrimers in circulation. It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons. It can bind to, and thus functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. Mouse and human TNF-alpha share 79% amino acid sequence identity. Unlike human TNF-alpha, the mouse form is glycosylated. In mouse deficiency of this gene is associated with defects in response to bacterial infection, with defects in forming organized follicular dendritic cell networks and germinal centers, and with a lack of primary B cell follicles.

2. Principle



An anti-tag antibody is pre-coated onto the bottom of wells. After adding antigen or samples, Tagged-Capture antibody and HRP-conjugated detection antibody, a sandwich complex is formed in the solution. TMB acts as a HRP substrate, and the solution color will change from colorless to blue. A stop solution containing sulfuric acid turns the solution yellow. The color intensity is proportional to the quantity of bound protein, which is measurable at 450 nm with the correction wavelength set at 630 nm.

3. Required Materials

3.1 A microplate reader capable of measuring absorbance at 450 nm with the correction wavelength set at 630 nm.

3.2 Calibrated, adjustable precision pipettes and disposable plastic tips. A manifold multi-channel pipette is recommended for large assays.

3.3 Plate washer: automated or manual.

3.4 Absorbent paper towels.

3.5 Glass or plastic tubes to prepare standard and sample dilutions.

3.6 Beakers and graduated cylinders.

3.7 Log-log or semi-log graph paper or computer and software for ELISA data analysis. A four-parameter logistic (4-PL) curve-fit is recommended. Proteintech data analysis website, https: //www.ptgcn.com/products/elisa-kits/.

3.8 Microplate thermostatic shaker.

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4. Kit Components and Storage

Microplate - 96 well microplate precoated an anti-tag antibody (8 well × 12 strips)	1 plate	**Unopened Kit:** Store at 2-8°C for 6 months or -20°C for 12 months. **Opened Kit:** All reagents stored at 2-8°C for 7 days. **Please use a new standard for each assay.**
Protein standard - 250 pg/bottle; lyophilized	2 bottles	
Capture antibody (100×) - 60 µL/vial*	1 vial	
Detection antibody, HRP-conjugated (100×) - 60 µL/vial*	1 vial	
Additional Diluent AT-60082 - 6 mL/bottle. Only for serum and plasma samples.	1 bottle	
Sample Diluent PT 3-ec - 30 mL/bottle. For mouse serum and plasma.	1 bottle	
Sample Diluent PT 4B1 - 30 mL/bottle. For cell culture supernatant.	1 bottle	
Detection Diluent - 15 mL/bottle	1 bottle	
Wash Buffer Concentrate (20×) - 30 mL/bottle	1 bottle	
Tetramethylbenzidine Substrate (TMB) - 12 mL/bottle	1 bottle	
Stop Solution - 12 mL/bottle	1 bottle	
Plate Cover Seals	4 pieces	

* Centrifugation immediately before use

5. Safety Notes

5.1 Avoid any skin and eye contact with Stop Solution and TMB. In case of contact, wash thoroughly with water.

5.2 Do not use the kit after the expiration date.

5.3 Do not mix or substitute reagents or materials from other kit lots or other sources.

5.4 Be sure to wear protective equipment such as gloves, masks and goggles during the experiment.

5.5 When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer to improve assay precision

6. Sample Collection and Storage

6.1 Serum: Allow blood samples to clot for 30 minutes, followed by centrifugation for 15 minutes at 1000xg. Clear serum can be assayed immediately or aliquoted and stored at -20°C. Avoid repeated freeze-thaw cycles.

6.2 Plasma: Use EDTA, heparin, or citrate as an anticoagulant for plasma collection. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. The plasma can be assayed immediately or aliquoted and stored at -20°C. Avoid repeated freeze-thaw cycles.

6.3 Cell Culture Supernatant: Remove particulates by centrifugation for 5 minutes at 500xg and assay immediately or aliquot and store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

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7. Regent Preparation

7.1 Wash Buffer (1X): If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 30 mL of Wash Buffer Concentrate(20X) to 570 mL deionized or distilled water to prepare 1X Wash Buffer.

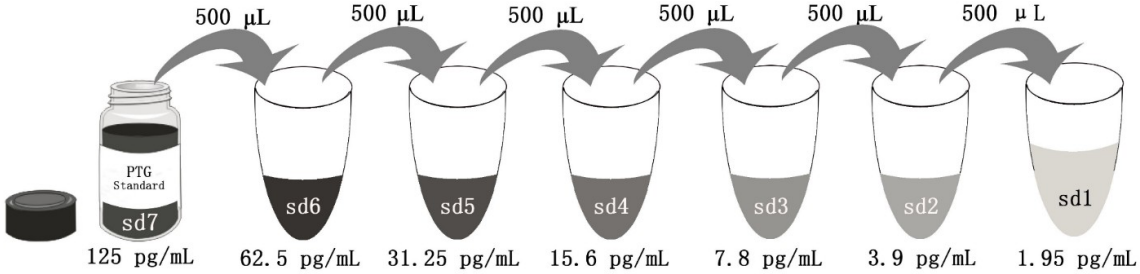
7.2 Antibody Cocktail (1X): Dilute 100X capture antibody and 100X HRP-conjugated detection antibody using Detection Diluent prior to assay. Suggested 1:100 dilution: 50 µL 100X capture antibody + 50 µL 100X Detection Antibody, HRP-conjugated + 4,900 µL Detection Diluent. Mix gently but thoroughly.

7.3 Sample Dilution: Different samples should be diluted with corresponding Sample Diluent, samples may require further dilution if the readout values are higher than the highest standard OD reading. Variations in sample collection, processing and storage may affect the results of the measurement.

Recommended Dilution for different sample types: 1:2 or 1:4 is recommended for mouse serum and plasma; 1:2 to 1:200 is recommended for cell culture supernatant.

7.4 Standard Serial Dilution:

For mouse serum and plasma, add 2 mL Sample Diluent PT 3-ec in protein standard. For cell culture supernatant, add 2 mL Sample Diluent PT 4B1 in protein standard.



Add # µL of Standard diluted in the previous step	—	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL
# µL of Sample Diluent PT 3-ec or PT 4B1	**2000 µL**	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL
	"sd7"	"sd6"	"sd5"	"sd4"	"sd3"	"sd2"	"sd1"

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8. Assay Procedure Summary

Bring all reagents to room temperature before use (Detection antibody, HRP-conjugated antibody can be used immediately). 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.

8.1 Preset the layout of the microplate, including control group, standard group and sample group, take out the required number of microplate strips and return excess strips to the foil pouch containing the drying reagent pack and reseal; store at 4°C immediately. Microplate strips should be used in one week.

8.2 Preset the layout of the microplate, including control group, standard group and sample group:

For serum or plasma, add 25 µL of Additional Diluent to the appropriate wells (No need incubation and wash);

For cell culture supernatant, no need to add Additional Diluent, directly follow the next step.

8.3 Add 50 µL standard or sample to appropriate wells. To avoid high background always add samples or standards to the well before the addition of antibody cocktail.

8.4 Add 50 µL 1× Antibody Cocktail solution (refer to Reagent Preparation 7.2) to each well. Seal plate with cover seal and incubate at 37°C on a microplate thermostatic shaker set at 400 rpm for 1 hour (incubate at 37°C for 2 hours is recommended if thermostatic shaker is not available) .

8.5 Wash

1) Gently remove the cover seal. Discard the liquid from wells by aspirating or decanting. Remove any residual solution by tapping the plate a few times on fresh paper towels.

2) Wash 4 times with 1× Wash Buffer, using at least 350-400 µL per well. Following the last wash, firmly tap plates on fresh towels 10 times to remove residual Wash Buffer. Avoid getting any towel fibers in the wells or wells drying out completely.

8.6 Add 100 µL TMB substrate solution to each well, protected from light. Incubate at 37°C on a microplate thermostatic shaker set at 400 rpm for 15 to 20 minutes. (Substrate Solution should remain colorless until added to the plate.)

8.7 Add 100 µL Stop Solution to each well in the same order as addition of the TMB substrate. Note: Avoid skin and eye contact with the Stop solution.

8.8 Read results immediately on a microplate reader at a wavelength of 450 nm. If possible, perform a double wavelength readout (450 nm and 630 nm).

8.9 Data analysis: Calculate the average of the duplicate readings (OD value) for each standard and sample, and subtract the average of the zero standard absorbance. Construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, four-parameter logistic curve- fit (4-PL) analysis is recommend. If the samples have been diluted, the fitting result must be multiplied by the dilution factor used.

Procedure summary

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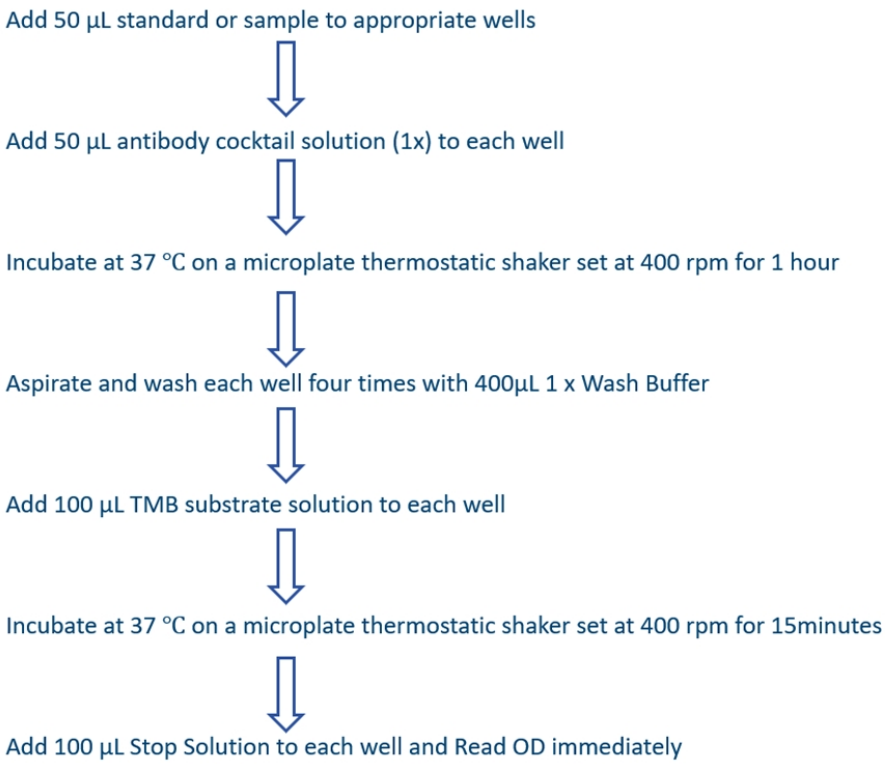

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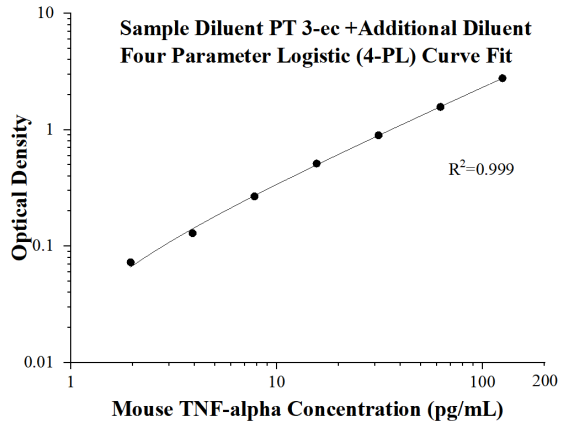
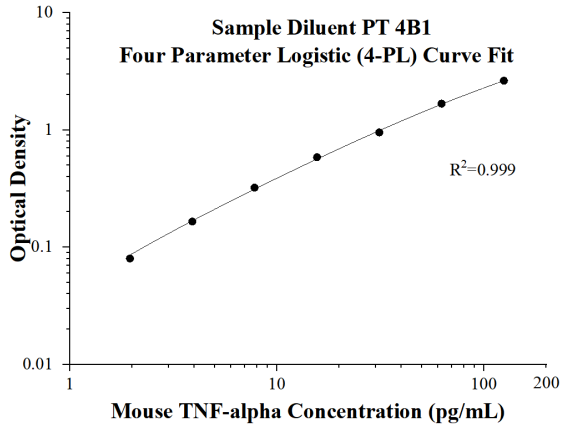
9. Validation Data

9.1 Standard curve

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



(pg/mL)	O.D	Average	Corrected
0	0.0385 0.041	0.0398	-
1.95	0.1141 0.1109	0.1125	0.0728
3.9	0.1748 0.163	0.1689	0.1292
7.8	0.3044 0.3099	0.3072	0.2674
15.6	0.5559 0.5491	0.5525	0.5128
31.25	0.9484 0.9261	0.9373	0.8975
62.5	1.5111 1.7097	1.6104	1.5707
125	2.8041 2.8272	2.8157	2.7759



(pg/mL)	O.D	Average	Corrected
0	0.1046 0.104	0.1043	-
1.95	0.1909 0.1777	0.1843	0.0800
3.9	0.2584 0.2808	0.2696	0.1653
7.8	0.4804 0.372	0.4262	0.3219
15.6	0.7103 0.6683	0.6893	0.5850
31.25	1.0337 1.0787	1.0562	0.9519
62.5	1.7659 1.7963	1.7811	1.6768
125	2.7137 2.7534	2.7336	2.6293

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9.2 Precision

Intra-assay Precision (Precision within an assay) Three samples of known concentration were tested 8 times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays) Three samples of known concentration were tested in 16 separate assays to assess inter-assay precision.

Intra-assay Precision				
Sample	n	Mean (pg/mL)	SD	CV%
1	8	70.6	2.1	3.0
2	8	15.1	0.5	3.3
3	8	7.0	0.4	5.7

Inter-assay Precision				
Sample	n	Mean (pg/mL)	SD	CV%
1	16	68.0	3.3	4.9
2	16	14.8	0.6	4.1
3	16	7.0	0.6	8.6

9.3 Recovery

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

Sample Type		Average% of Expected	Range (%)
Mouse serum	1:2	104	90-116
Cell culture supernatant	1:2	116	106-122

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9.4 Sample values

Mouse serum - mouse serum samples were evaluated for the presence of mouse TNF-alpha in this assay.

Sample Type	Mean (pg/mL)	Range (pg/mL)
Mouse serum (n=16)	10.8	4.9-20.5

Four mice were each injected with 15 µg of LPS and were bled 2 hours after injection. Mouse serum samples were evaluated for the presence of mouse TNF-alpha in this assay.

Sample Type	Mean (pg/mL)	Range (pg/mL)
Mouse serum (n=4)	467.1	351.3-581.6

Cell culture supernatant:

Organs from mice were rinsed with PBS to remove excess blood, homogenized with a tissue homogenizer, and 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 µg/mL LPS for 1 day or 3 days. Aliquots of the cell culture supernatants were removed and assayed for levels of mouse TNF-alpha.

Tissue Type	Unstimulated (pg/mL)	Stimulated (pg/mL)
Mouse lung (1 day)	0.2	67.5
Mouse spleen (3 days)	2.5	19.8

RAW 264.7 mouse mononuclear macrophages cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2.5 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 1 µg/mL LPS for 4 days. Aliquots of the cell culture supernatants were removed and assayed for levels of mouse TNF-alpha.

Condition	Day 4 (pg/mL)
Unstimulated	230.2
Stimulated	9,609.7

9.5 Sensitivity

The minimum detectable dose of mouse TNF-alpha is 0.2 pg/mL. This was determined by adding two standard deviations to the concentration corresponding to the mean O.D. of 20 zero standard replicates.

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9.6 Linearity

To assess the linearity of the assay, mouse serum samples were spiked with high concentrations of mouse TNF-alpha and diluted with the appropriate **Sample Diluent** to produce samples with values within the dynamic range of the assay. Cell culture supernatant samples were diluted with the appropriate **Sample Diluent** to produce samples with values within the dynamic range of the assay.

(The cell culture supernatant was initially diluted 1:50.)

		Mouse serum (Sample Diluent PT 3-ec)	Cell culture supernatant (Sample Diluent PT 4B1)
1:2	Average% of Expected	104	100
	Range (%)	97-111	-
1:4	Average% of Expected	104	96
	Range (%)	95-113	95-97
1:8	Average% of Expected	100	76
	Range (%)	97-103	75-77
1:16	Average% of Expected	102	86
	Range (%)	101-103	72-100

9.7 Specificity

This assay recognizes natural and recombinant mouse TNF-alpha.

The following factors prepared at 50 ng/mL were assayed and exhibited no cross-reactivity or interference.

Recombinant mouse:

CD40

Fas

CD40 Ligand

TNF-β

LIF

A sample containing 50 ng/mL of the recombinant human TNF-alpha reads as 28.4 pg/mL (0.06% cross-reactivity).

10. References

1. Agbanoma, Gideon et al. Journal of immunology (Baltimore, Md. : 1950) vol. 188,3 (2012): 1307-17.

2. Kriegler, M et al. Cell vol. 53,1 (1988): 45-53.

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5. Locksley, R M et al. Cell vol. 104,4 (2001): 487-501.

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