

Version 2 Last updated 20 August 2019

ab208348 Mouse TNF alpha SimpleStep ELISA[®] Kit

For the quantitative measurement of mouse TNF alpha in serum, plasma, and cell culture supernatants.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Abcam's TNF alpha in vitro SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of TNF alpha protein in mouse serum, plasma, and cell culture supernatants.

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB substrate is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

Tumor necrosis factor, or TNF alpha, is a cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFR. Furthermore, TNF alpha is a ligand of the TNF superfamily which plays a central role in inflammation, apoptosis, proliferation, invasion, angiogenesis, metastasis, and morphogenesis. It is mainly secreted by macrophages, and can induce cell death of certain tumor cell lines. Rat and Human TNF alpha are 95% and 79% identical to Mouse TNF alpha, respectively.

TNF alpha is expressed on macrophages and endothelial, epithelial, and tumor cells as a 26 kDa transmembrane protein. TNF-alpha is cleaved by proteolytic processing into six chains: (1) TNF membrane form, (2) Intracellular domain 1 (ICD1), (3) Intracellular domain 2 (ICD2), (4) C-domain 1, (5) C-domain 2 and (6) TNF soluble form. ICD1 and ICD2 are released into the cytosol, while C-domain 1 and C-domain 2 are released into the extracellular space. Furthermore, the membrane form, but not the soluble form, is phosphorylated on serine residues.

Dephosphorylation of the membrane form occurs by binding to soluble TNFRSF1A/TNFR1.

Signaling from TNF-alpha differs depending on the type of ligand initiating the signaling event (intracellular, membrane, or soluble). As an example, the membrane form of TNF-alpha appears to mediate anti-tumorigenic therapeutic responses whereas the soluble ligand is linked to inflammation and proliferation. Likewise, the TNF intracellular domain (ICD) form induces IL-12 production in dendritic cells.

2. Protocol Summary

Prepare all reagents, samples, and standards as instructed



Add 50 μ L standard or sample to appropriate wells



Add 50 μ L Antibody Cocktail to all wells



Incubate at room temperature for 1 hour



Aspirate and wash each well three times with 350 μ L 1X Wash Buffer PT



Add 100 μ L TMB Development Solution to each well and incubate for 10 minutes.



Add 100 μ L Stop Solution and read OD at 450 nm

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted. Refer to list of materials supplied for storage conditions of individual components.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition
10X Mouse TNF alpha Capture Antibody	600 μ L	4°C
10X Mouse TNF alpha Detector Antibody	600 μ L	4°C
Mouse TNF alpha Lyophilized Recombinant Protein	2 Vials	4°C
Antibody Diluent CPI	6 mL	4°C
10X Wash Buffer PT	20 mL	4°C
TMB Substrate	12 mL	4°C
Stop Solution	12 mL	4°C
Sample Diluent NS	50 mL	4°C
Sample Diluent NBS	20 mL	4°C
Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	4°C
Plate Seal	1	4°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

8. Technical Hints

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- The provided Antibody Diluents and Sample Diluents contain protease inhibitor aprotinin. Additional protease inhibitors can be added if required.
- **To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.**
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

9.1 Sample Diluent 25BS

Prepare Sample Diluent 25BS by diluting Sample Diluent NBS with Sample Diluent NS. To make 10 mL Sample Diluent 25BS combine 2.5 mL Sample Diluent NBS with 7.5 mL Sample Diluent NS.

9.2 Sample Diluent 10BS

Prepare Sample Diluent 10BS by diluting Sample Diluent NBS with Sample Diluent NS. To make 10 mL Sample Diluent 10BS combine 1 mL Sample Diluent NBS with 9 mL Sample Diluent NS.

9.3 1X Wash Buffer PT

Prepare 1X Wash Buffer PT by diluting 10X Wash Buffer PT with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL 10X Wash Buffer PT with 45 mL deionized water. Mix thoroughly and gently.

9.4 Antibody Cocktail

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent CPI. To make 3 mL of the Antibody Cocktail combine 300 μ L 10X Capture Antibody and 300 μ L 10X Detector Antibody with 2.4 mL Antibody Diluent CPI. Mix thoroughly and gently.

10. Standard Preparation

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of positive controls for every use.

The following section describes the preparation of a standard curve for duplicate measurements (recommended).

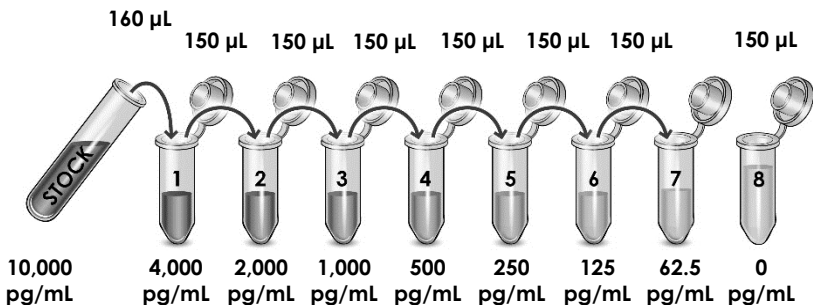
10.1 For **serum and plasma heparin samples** follow these instructions:

10.1.1 Reconstitute the mouse TNF alpha standard sample by adding 1 mL Sample Diluent 25BS. Mix thoroughly and gently. Hold at room temperature for 10 minutes and mix gently. This is the 10,000 pg/mL **Stock Standard Solution**.

10.1.2 Label eight tubes, Standards 1– 8.

10.1.3 Add 240 μ L Sample Diluent 25BS into tube number 1 and 150 μ L of Sample Diluent 25BS into numbers 2-8.

10.1.4 Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:



10.2 For **plasma citrate and EDTA samples and cell culture supernatant samples**, follow these instructions:

10.2.1 For **plasma citrate and EDTA samples** reconstitute the mouse TNF alpha standard sample by adding 1 mL Sample Diluent 10BS.

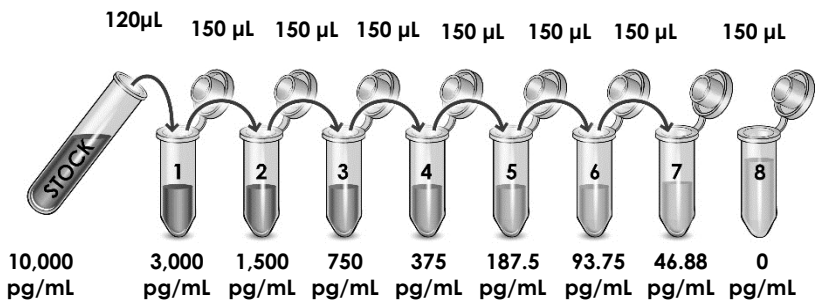
For **cell culture supernatant samples** reconstitute the mouse TNF alpha standard sample by adding 1 mL Sample Diluent NS.

Mix thoroughly and gently. Hold at room temperature for 10 minutes and mix gently. This is the 10,000 pg/mL **Stock Standard Solution**.

10.2.2 Label eight tubes, Standards 1–8.

10.2.3 Add 280 μ L appropriate sample diluent (see Step 10.2) into tube number 1 and 150 μ L of appropriate sample diluent into numbers 2-8.

10.2.4 Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:



11. Sample Preparation

TYPICAL SAMPLE DYNAMIC RANGE	
Sample Type	Range (%)
Mouse Plasma - Heparin	6.25 – 100
Mouse Plasma - EDTA	6.25 – 100
Mouse Plasma - Citrate	6.25 – 100
Mouse Serum	6.25 – 100
RAW 264.7 LPS Stimulated Supernatant	12.5 – 50
Cell Culture Media	6.25 – 100

11.1 Plasma

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute citrate and EDTA samples into Sample Diluent 10BS and assay. Dilute heparin samples into Sample Diluent 25BS and assay. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.2 Serum

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples into Sample Diluent 25BS and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

11.3 Cell Culture Supernatants

Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants and dilute samples into Sample Diluent NS and assay. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or “edge effects” have not been observed with this assay.

13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
 - We recommend that you assay all standards, controls and samples in duplicate.
- 13.1 Prepare all reagents, working standards, and samples as directed in the previous sections.
 - 13.2 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.
 - 13.3 Add 50 µL of all sample or standard to appropriate wells.
 - 13.4 Add 50 µL of the Antibody Cocktail to each well.
 - 13.5 Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.
 - 13.6 Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.
 - 13.7 Add 100 µL of TMB Substrate to each well and incubate for 20 minutes in the dark on a plate shaker set to 400 rpm.
 - 13.8 Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.

Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

Mode:	Kinetic
Wavelength:	600 nm
Time:	up to 15 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

Note that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 μ L Stop Solution to each well and recording the OD at 450 nm.

13.9 Analyze the data as described below.

14. Calculations

14.1 Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control

standard absorbance value from all other absorbance values.

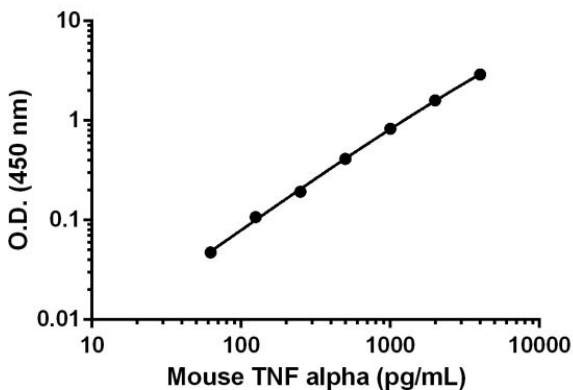
- 14.2 Create a standard curve by plotting the average blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.

Note: Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4 parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.

- 14.3 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted absorbance values against the standard curve. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.
- 14.4 Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

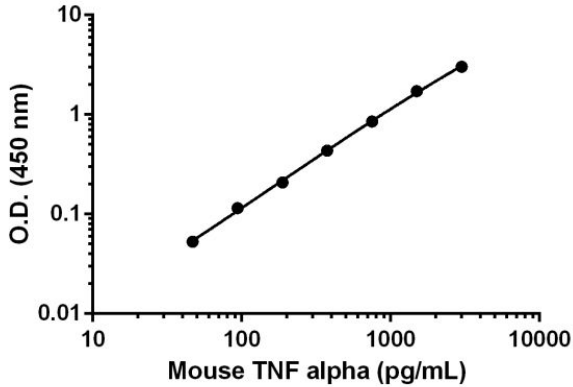
15. Typical Data

Typical standard curve – data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.



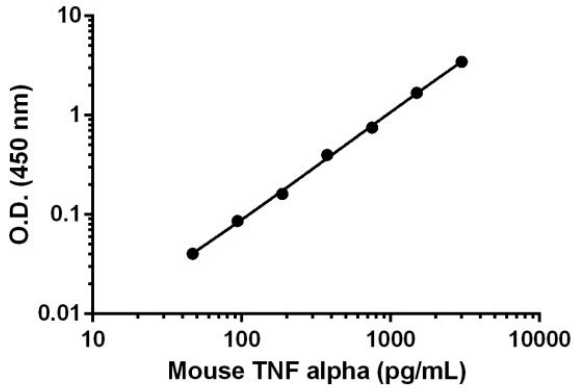
Standard Curve Measurements			
Conc. (pg/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.054	0.066	0.060
62.5	0.107	0.107	0.107
125	0.172	0.163	0.167
250	0.257	0.250	0.253
500	0.481	0.468	0.474
1,000	0.904	0.879	0.891
2,000	1.693	1.633	1.663
4,000	3.001	2.924	2.962

Figure 1. Example of mouse TNF alpha standard curve in Sample Diluent 25BS. The TNF alpha standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.



Standard Curve Measurements			
Conc. (pg/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.088	0.070	0.079
46.88	0.132	0.132	0.132
93.75	0.187	0.202	0.194
187.5	0.280	0.293	0.287
375	0.519	0.511	0.515
750	0.934	0.935	0.935
1,500	1.797	1.806	1.801
3,000	3.098	3.116	3.107

Figure 2. Example of mouse TNF alpha standard curve in Sample Diluent 10BS. The TNF alpha standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.



Standard Curve Measurements			
Conc. (pg/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.070	0.076	0.073
46.88	0.131	0.132	0.132
93.75	0.198	0.183	0.191
187.5	0.306	0.309	0.308
375	0.520	0.537	0.529
750	0.965	0.963	0.964
1,500	1.840	1.821	1.830
3,000	3.046	3.134	3.090

Figure 3. Example of mouse TNF alpha standard curve in Sample Diluent NS. The TNF alpha standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. Calibration

This immunoassay is calibrated against a highly purified mouse TNF alpha. The NIBSC/WHO unclassified purified mouse TNF alpha preparation 88/532 was evaluated in this kit.

The dose response curve of the unclassified standard 88/532 parallels the SimpleStep standard curve. To convert sample values obtained with the SimpleStep mouse TNF alpha kit to approximate NIBSC 88/532 units, use the equation below.

NIBSC (88/532) approximate value (units/mL) = 2.0 x SimpleStep mouse TNF alpha value (pg/mL)

17. Typical Sample Values

SENSITIVITY –

The calculated minimal detectable dose (MDD) is determined by calculating the mean of zero standard replicates and adding 2 standard deviations then extrapolating the corresponding concentrations. The MDD is dependent on the Sample Diluent buffer used:

Sample Diluent Buffer	n=	Minimal Detectable Dose
Sample Diluent NS	24	9.8 pg/mL
Sample Diluent 10BS	24	12.1 pg/mL
Sample Diluent 25BS	24	9.1 pg/mL

RECOVERY –

Three concentrations of mouse TNF alpha were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
Mouse Plasma – Heparin	92	85 – 103
Mouse Plasma – EDTA (100%)	99	98 – 101
Mouse Plasma – Citrate (100%)	87	85 – 88
Mouse Serum (100%)	97	85 – 115
Cell Culture Media (100%)	111	109 – 115

Linearity of Dilution

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Native mouse TNF alpha was measured in RAW 264.7 LPS stimulated media in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS. Recombinant mouse TNF alpha was spiked into cell culture media and diluted in a 2-fold dilution series in Sample Diluent NS.

Dilution Factor	Interpolated value	50% RAW 264.7 LPS Stimulated Media	100% Cell Culture Media
Undiluted	pg/mL	3,173.3	1,010.3
	% Expected value	100	100
2	pg/mL	1619.1	512.1
	% Expected value	102	101
4	pg/mL	673.6	258.3
	% Expected value	85	102
8	pg/mL	NL	135.5
	% Expected value	NL	107
16	pg/mL	NL	55.6
	% Expected value	NL	88

NL – Non-Linear

Recombinant mouse TNF alpha was spiked into mouse serum and mouse heparin plasma and diluted in a 2-fold dilution series in Sample Diluent 25BS. Recombinant mouse TNF alpha was spiked into mouse citrate and mouse EDTA plasmas and diluted in a 2-fold dilution series in Sample Diluent 10BS.

Dilution Factor	Interpolated value	100% Mouse Serum	100% Mouse Plasma (Citrate)	100% Mouse Plasma (EDTA)	100% Mouse Plasma (Heparin)
Undiluted	pg/mL	1,724.5	1,750.2	2,019.5	1,544.2
	% Expected value	100	100	100	100
2	pg/mL	832.3	832.8	922.0	705.4
	% Expected value	97	95	91	91
4	pg/mL	384.8	424.9	444.4	357.7
	% Expected value	89	97	88	93
8	pg/mL	191.5	206.8	214.3	178.8
	% Expected value	89	95	85	93
16	pg/mL	104.6	101.1	104.4	94.4
	% Expected value	97	92	83	98

PRECISION –

Mean coefficient of variations of interpolated values of TNF alpha in 3 concentrations of RAW 264.7 LPS stimulated media within the working range of the assay.

	Intra-Assay	Inter-Assay
n =	8	3
CV(%)	6.7	9.8

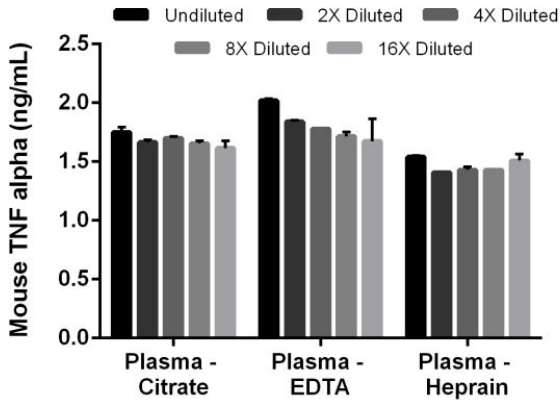
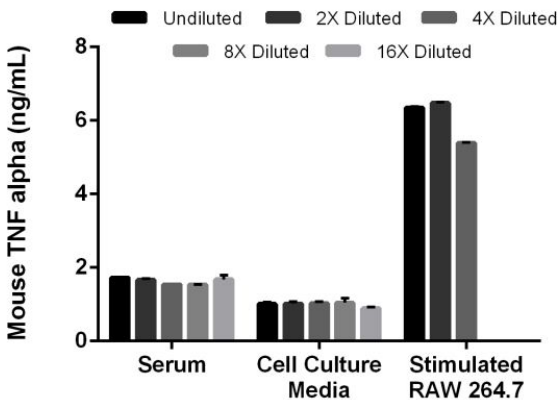


Figure 4.

Interpolated concentrations of spiked TNF alpha in mouse plasma samples. The concentrations of TNF alpha were measured in duplicates, interpolated from the TNF alpha standard curves and corrected for sample dilution. Undiluted samples are as follows: plasma (citrate) 100%, plasma (EDTA) 100%, and plasma (heparin) 100%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean TNF alpha concentration was determined to be 1.68 ng/mL in plasma (citrate), 1.81 ng/mL in plasma (EDTA) and 1.47 ng/mL in plasma (heparin).



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Figure 5. Interpolated concentrations of spiked mouse TNF alpha in mouse serum and cell culture samples and native mouse TNF alpha in mouse RAW 264.7 LPS stimulated cell culture supernatants. The concentrations of TNF alpha were measured in duplicates, interpolated from the TNF alpha standard curves and corrected for sample dilution. Undiluted samples are as follows: serum 100%, cell culture 100%, and RAW 264.7 LPS stimulated cell culture supernatants 50%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean TNF alpha concentration was determined to be 1.63 ng/mL in serum, 1.00 ng/mL in cell culture and 6.07 ng/mL in RAW 264.7 LPS stimulated cell culture supernatants.

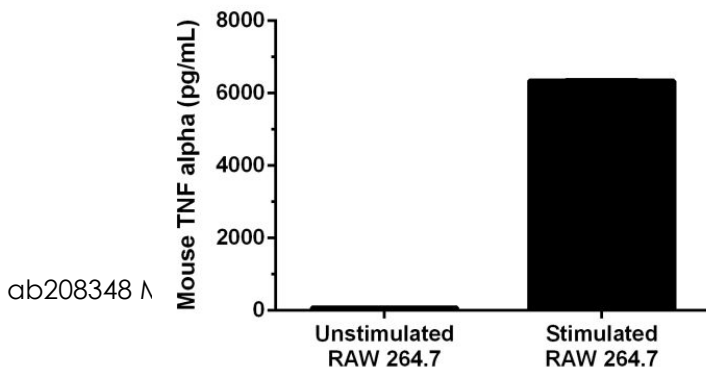


Figure 6. Interpolated concentrations of native TNF alpha in mouse RAW 264.7 LPS stimulated and unstimulated cell culture supernatant samples. The concentrations of TNF alpha were measured in duplicates, interpolated from the TNF alpha standard curves and corrected for sample dilution. Undiluted samples are as follows: RAW 264.7 LPS stimulated supernatant 50% and RAW 264.7 LPS unstimulated supernatant 50%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean TNF alpha concentration was determined to be 6346.4 pg/mL in RAW 264.7 LPS stimulated supernatant and 83.7 pg/mL in RAW 264.7 LPS unstimulated supernatant. RAW 264.7 cells were cultured in HGDMEM with 100 µg/mL Kanamycin and 2 mM L-glutamine. Cells were starved for 24 hours and treated in the presence and absence of 5 µg/mL of LPS.

18. Assay Specificity

This kit recognizes both native and recombinant mouse TNF alpha protein in serum, plasma, and cell culture supernatant samples only.

CROSS REACTIVITY

Recombinant mouse TNF RI, TNF RII, OPG, CD40 receptor, IL1 beta, IL-16, and IL-5 were prepared at 100 ng/mL and assayed for cross reactivity. No cross-reactivity was observed.

Recombinant human TNF alpha was prepared at 100 ng/mL, 25 ng/mL, and 3 ng/mL and assayed for cross reactivity. On average, 3.0% cross-reactivity was observed with a standard deviation of 0.7%.

INTERFERENCE

Recombinant mouse TNF RI, TNF RII, OPG, CD40 receptor, IL1 beta, IL-16, and IL-5 and recombinant human TNF alpha were prepared at 100 ng/mL and tested for interference. No interference with was observed.

19. Species Reactivity

This kit recognizes mouse TNF alpha protein.

Please contact our Technical Support team for more information.

20. Troubleshooting

Problem	Reason	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop solution
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB substrate solution protected from light.
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

21. Notes

Technical Support

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