

Version 1 Last updated 25 June 2019

ab181421 Human TNF alpha SimpleStep ELISA[®] Kit

For the quantitative measurement of TNF alpha in human serum, plasma, and cell culture supernatant.

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

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1. Overview

TNF alpha *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of TNF alpha protein in human serum, plasma, and cell culture supernatant.

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 Development Solution 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.

TNF alpha, also known as cachectin or TNFSF1A, is the prototypic ligand of the TNF superfamily which plays a central role in inflammation, apoptosis, proliferation, invasion, angiogenesis, metastasis and morphogenesis. It is expressed on macrophages, endothelial, epithelial and tumor cells as a 26kDa transmembrane protein. TNF alpha is cleaved by proteolytic processing into six chains: (1) TNF membrane form, (2) Intracellular domain 1, (3) Intracellular domain 2, (4) C-domain 1, (5) C-domain 2 and (6) TNF soluble form. 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.

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
Human TNF alpha Capture Antibody 10X	600 µL	+4°C
Human TNF alpha Detector Antibody 10X	600 µL	+4°C
Human TNF alpha Lyophilized Recombinant Protein	2 Vials	+4°C
Antibody Diluent 4BR	6 mL	+4°C
Wash Buffer PT 10X	20 mL	+4°C
TMB Development Solution	12 mL	+4°C
Stop Solution	12 mL	+4°C
Sample Diluent NS	50 mL	+4°C
SimpleStep Pre-Coated 96-Well Microplate	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 perform 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 1X Wash Buffer PT:

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

9.2 Antibody Cocktail:

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 4BR. 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 4BR. Mix thoroughly and gently.

10. Standard Preparation

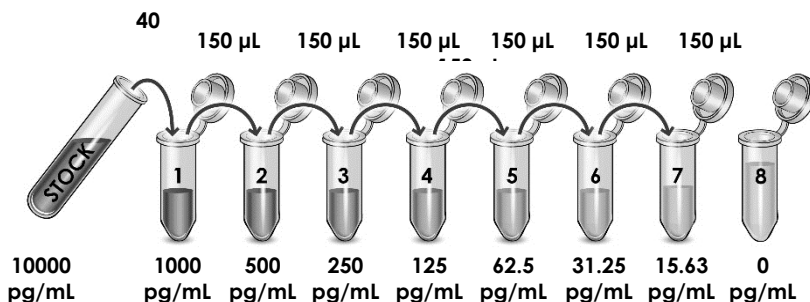
- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

10.1 Reconstitute the TNF alpha standard sample by adding 1000 μL Sample Diluent NS. Mix thoroughly and gently. Hold at room temperature for 10 minutes and mix gently. This is the 10000 pg/mL **Stock Standard** Solution.

10.1.1 Label eight tubes, Standards 1– 8.

10.1.2 Add 360 μL Sample Diluent NS into tube number 1 and 150 μL of Sample Diluent NS into numbers 2-8.

10.1.3 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
Serum*	≤ 1:40
Plasma – Citrate*	≤ 1:40
Plasma – EDTA*	≤ 1:40
Plasma – Heparin*	≤ 1:40
PBMC cell culture supernatant**	1:80 -1:10

*Based on spiked sample

**Range can vary depending on stimulation factors.

11.1 Plasma:

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples at least 1:40 into Sample Diluent NS 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 at least 1:40 into Sample Diluent NS 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 assay. Or dilute samples at least 1:10 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 Development Solution to each well and incubate for 10 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.
 - 13.9** 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

Δ 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.

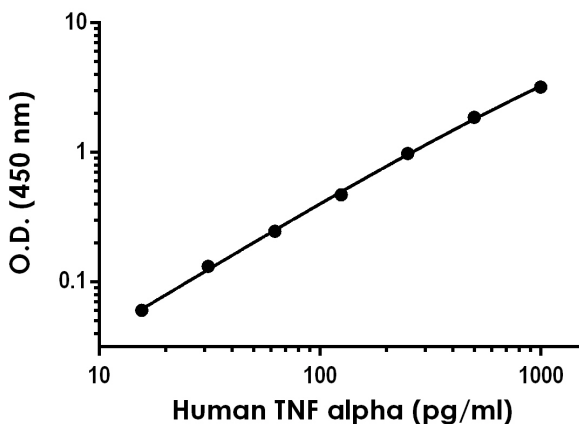
13.10 Analyze the data as described below.

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.
 - Δ **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			
Concentration (pg/mL)	O.D 450 nm		Mean O.D
	1	2	
0	0.061	0.068	0.065
15.63	0.127	0.123	0.125
31.25	0.189	0.206	0.197
62.5	0.312	0.312	0.312
125	0.555	0.521	0.538
250	1.065	1.030	1.048
500	1.925	1.941	1.933
1000	3.362	3.161	3.262

Figure 1. Example of human 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 human TNF alpha. The NIBSC/WHO unclassified purified human TNF alpha preparation 12/154 was evaluated in this kit.

The dose response curve of the unclassified standard TNF alpha parallels the SimpleStep standard curve. To convert sample values obtained with the SimpleStep Human TNF alpha kit to approximate NIBSC 12/154 International units, use the equation below.

NIBSC (12/154) approximate value (IU/mL) = 0.094 x SimpleStep Human TNF alpha value (pg/mL).

17. Typical Sample Values

SENSITIVITY –

The calculated minimal detectable dose (MDD) is 4.32 pg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=24) and adding 2 standard deviations then extrapolating the corresponding concentration.

RECOVERY –

Three concentrations of TNF alpha recombinant protein 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 (%)
2.5% Serum	88	83-93
2.5% Plasma-Citrate	91	86-97
2.5% Plasma-EDTA	100	95-104
2.5% Plasma-Heparin	98	89-110
5% PHA-M treated PBMC Cell culture supernatant*	110	105-113

*Media is RPMI 1640 containing 10% fetal calf serum.

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 TNF alpha was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	10% PHA-M Treated PBMC Cell Culture Supernatant
Undiluted	pg/mL	726.56
	% Expected value	100
2	pg/mL	355.55
	% Expected value	98
4	pg/mL	183.66
	% Expected value	101
8	pg/mL	94.78
	% Expected value	104

Recombinant TNF alpha was spiked into the following biological samples and diluted in a 2-fold dilution series in Sample Diluent NS.

Dilution Factor	Interpolated value	2.5% Human Serum	2.5% Human Plasma (Citrate)	2.5% Human Plasma (EDTA)	2.5% Human Plasma (Heparin)
Undiluted	pg/mL	447.20	451.09	483.71	499.11
	% Expected value	100	100	100	100
2	pg/mL	223.35	234.34	244.59	237.17
	% Expected value	100	104	101	95
4	pg/mL	119.50	127.27	123.94	120.73
	% Expected value	107	113	102	97
8	pg/mL	60.23	59.54	62.05	60.11
	% Expected value	108	106	103	96
16	pg/mL	28.78	NL	NL	NL
	% Expected value	103	NL	NL	NL

NL – Non-Linear

Twenty individual healthy human female/male donors were measured in duplicate for the presence of TNF alpha. All values were below the detectable range of the assay.

PRECISION –

Mean coefficient of variations of interpolated values of TNF alpha from a single concentration of PHA-M treated PBMC cell culture supernatant within the working range of the assay.

	Intra- Assay	Inter- Assay
n =	8	3
CV(%)	2.5	3.1

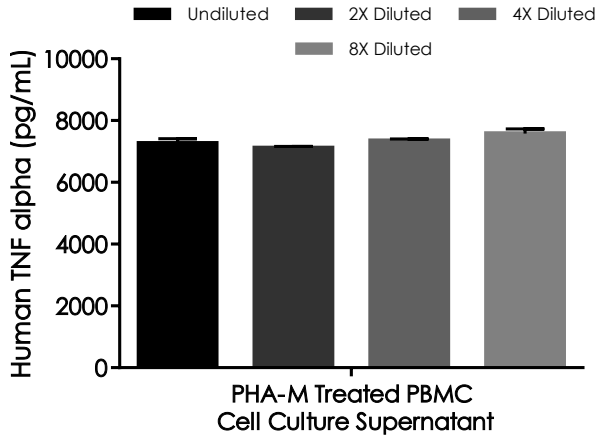


Figure 2. Interpolated concentrations of native TNF alpha in human PBMC cell culture supernatant samples. PBMC cells were treated with 1.5% PHA-M for 46 hours in 10F RPMI. The concentrations of TNF alpha were measured in duplicates, interpolated from the TNF alpha standard curve and corrected for sample dilution. Undiluted samples are PHA-M treated PBMC cell culture supernatant 10%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean TNF alpha concentration was determined to be 7326.31 pg/mL in PHA-M treated PBMC cell culture supernatant.

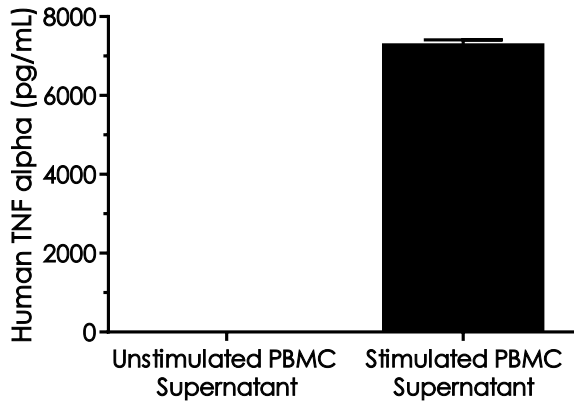


Figure 3. Interpolated concentrations of native TNF alpha PBMC cell culture supernatant samples. PBMC cells were stimulated with 1.5% PHA-M or vehicle control in 10F RPMI and incubated for 46 hours. The concentrations of TNF alpha were measured in duplicate and interpolated from the TNF alpha standard curves. Undiluted samples are PHA-M stimulated PBMC cell culture supernatant 10% and unstimulated PBMC cell culture supernatant 10%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean TNF alpha concentration was back calculated determined to be 7265.71 pg/mL in PHA-M stimulated PBMC cell culture supernatant and undetectable in the unstimulated PBMC control.

18. Assay Specificity

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

CROSS REACTIVITY

Recombinant proteins were prepared at 1000 pg/mL and assayed for cross reactivity. No cross reactivity was found for the following targets:

- Human IL-2
- Human IL-4
- Human IL-1 alpha
- Human IFN gamma
- Human TNF beta
- Human TNF R1

INTERFERENCE

Recombinant Human TNF R1 was prepared at 1000 pg/mL and tested for interference. No interference with was observed.

19. Species Reactivity

This kit recognizes human TNF alpha protein.

Other species reactivity was determined by measuring 1000 pg/mL recombinant proteins of various species, interpolating the protein concentrations from the human standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration assayed at the same dilution.

Reactive species:

- Primate

Reactivity < 3% was determined for the following species:

- Mouse
- Rat

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 Development 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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