



TNF ELISA Kit (Human) (OKBB00261)

Instruction for Use

For the quantitative measurement of Human TNF in cell culture supernatants, serum and plasma (heparin, EDTA, citrate).

Lot to lot variation can occur. Refer to the manual provided with the kit.

This product is intended for research use only.

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1. Background

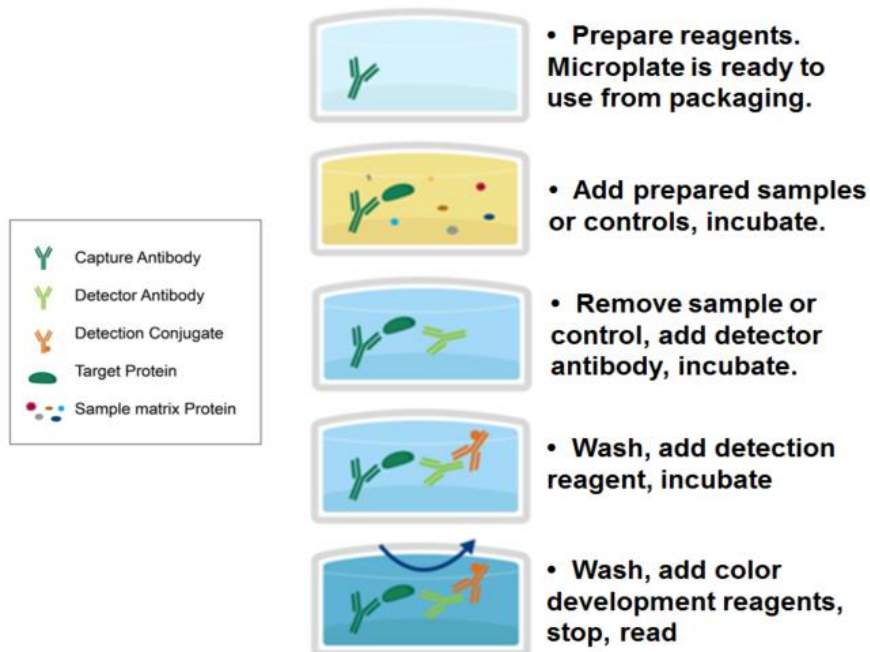
Principle

Aviva Systems Biology TNF ELISA Kit (Human) (OKBB00261) is based on standard sandwich enzyme-linked immune-sorbent assay technology. An antibody specific for TNF has been pre-coated onto 96-wellplate (12 x 8 Well Strips). Standards (E.coli,V77-L233) and test samples are added to the wells, incubated and removed. A biotinylated detector antibody specific for TNF is added, incubated and followed by washing. Avidin-Biotin-Peroxidase Complex is then added, incubated and unbound complex is washed away. An enzymatic reaction is visualized through the addition of TMB substrate which is catalyzed by HRP to produce a blue color product that changes to yellow after adding acidic stop solution. The density of yellow coloration read by absorbance at 450 nm and is quantitatively proportional to the amount of sample Human TNF captured in the well.

Background

Tumor necrosis factor-alpha (TNF-alpha, or TNF) is secreted by macrophages in response to inflammation, infection and cancer.¹ Human Tumor Necrosis Factor (TNF) and Lymphotoxin (TNF-beta) are cytotoxic proteins which have similar biological activities and share 30% amino acid homology.² TNF-alpha is produced by monocytes, which can stimulate endothelial cells to produce the multilineage growth factor granulocyte-macrophage colony-stimulating factor and extend the role of this immunoregulatory protein to the regulation of hematopoiesis in vitro.³ TNF is a soluble protein that causes damage to tumor cells but has no effect on normal cells. Human TNF has been purified to apparent homogeneity as a 17.3-kilodalton protein from HL-60 leukemia cells and has showed cytotoxic and cytostatic activities against various human tumor cell lines. The human TNF cDNA is 1585 base pairs in length and encodes a protein of 233 amino acids. The mature protein begins at residue 77, leaving a long leader sequence of 76 amino acids.⁴ TNF-alpha has been mapped to human chromosome 6.²

2. Assay Summary



3. Precautions

- Read instructions fully prior to beginning use of the assay kit.
- Any deviations or modifications from the described method or use of other reagents could result in a reduction of performance.
- Reduce exposure to potentially harmful substances by wearing personal protective lab equipment including lab coats, gloves and glasses.
- For information on hazardous substances included in the kit please refer to the Material Safety Data Sheet (MSDS).

4. Storage and Stability

- Upon receipt store kit at 4°C for 6 months or -20°C for 12 months. Avoid multiple freeze/thaw cycles.

5. Kit Components

- The following reagents are the provided contents of the kit.

Description	Quantity
96-Well plate Pre-coated with Anti-Human TNF Antibody	96 Wells (12 x 8)
Lyophilized Recombinant Human TNF standard	10 ng/tube x 2
100X Biotinylated Anti-Human TNF Antibody	130 µL
100X Avidin-Biotin-Peroxidase Complex (ABC)	130 µL
Sample Diluent Buffer	30 mL
Antibody Diluent Buffer	12 mL
ABC Diluent Buffer	12 mL
TMB Color Developing Agent	10 mL
TMB Stop Solution	10 mL
10X Wash Buffer	30 mL

6. Required Materials Not Supplied

- Microplate reader capable of reading absorbance at 450 nm.
- Automated plate washer (optional).
- Pipettes capable of precisely dispensing 0.5 µL through 1 mL volumes of aqueous solutions.
- Pipettes or volumetric glassware capable of precisely measuring 1 mL through 100 mL of aqueous solutions.
- New, clean tubes and/or micro-centrifuge tubes for the preparation of standards or samples.
- Absorbent paper or paper toweling.
- Distilled or deionized ultrapure water.

7. Technical Application Tips

- Do not mix or substitute components from other kits.
- To ensure the validity of experimental operation, it is recommended that pilot experiments using standards and a small selection of sample dilutions to ensure optimal dilution range for quantitation.
- Samples exhibiting OD measurements higher than the highest standard should be diluted further in the appropriate sample dilution buffers.
- Inspect all reagents prior to use. Components should contain no particulates or cloudiness and should be colorless.
- Prior to using the kit, briefly spin component tubes to collect all reagent at the bottom.
- Replicate wells are recommended for standards and samples.
- Cover microplate while incubating to prevent evaporation.
- Do not allow the microplate wells dry at any point during the assay procedure.
- Do not reuse tips or tube to prevent cross contamination.
- Avoid causing bubbles or foaming when pipetting, mixing or reconstituting.
- Completely remove of all liquids when washing to prevent cross contamination.
- Prepare reagents immediately prior to use and do not store, with the exception of the top standard.
- Equilibrate all materials to ambient room temperature prior to use (standards exception).
- For optimal results in inter- intra-assay consistency, equilibrate all materials to 37°C prior to performing assay (standards exception) and perform all incubations at 37°C.
- Pipetting less than 1 μ L is not recommended for optimal assay accuracy.
- Once the procedure has been started, all steps should be completed without interruption. Ensure that all reagents, materials and devices are ready at the appropriate time.
- Incubation times will affect results. All wells should be handled in the same sequential order and time intervals for optimal results.
- Samples containing precipitates or fibrin strands or which are hemolytic or lipemic might cause inaccurate results due to interfering factors.

8. Reagent Preparation

- Equilibrate all materials to room temperature prior to use and use prepare immediately prior to use.

8.1 **1X Biotinylated Anti-Human TNF Antibody**

- 8.1.1 Prepare the **1X Biotinylated Anti-Human TNF Antibody** immediately prior to use by diluting the **100X Biotinylated Anti-Human TNF Antibody** 1:100 with Antibody Diluent Buffer.
- 8.1.2 For every 8 wells to be used (one 8 well strip) in the experiment prepare 1,000 μ L by adding 10 μ L of **100X Biotinylated Anti-Human TNF Antibody** to 990 μ L **Antibody Diluent Buffer**.
- 8.1.3 Mix thoroughly and gently. Hold no longer than 2 hours prior to using in procedure.

8.2 **1X Avidin-Biotin-Peroxidase Complex (ABC)**

- 8.2.1 Prepare the **1X Avidin-Biotin-Peroxidase Complex (ABC)** immediately prior to use by diluting the **100X Avidin-Biotin-Peroxidase Complex (ABC)** 1:100 with **ABC Dilution Buffer**.
- 8.2.2 For every 8 wells to be used (one 8 well strip) in the experiment prepare 1,000 μ L, by adding 10 μ L of **100X Avidin-Biotin-Peroxidase Complex (ABC)** to 990 μ L **ABC Dilution Buffer**.
- 8.2.3 Mix thoroughly and gently. Hold no longer than 2 hours prior to using in procedure.

8.3 TNF Assay standards

- 8.3.1 Prepare the TNF standards no greater than 2 hours prior to performing experiment. standards should be held on ice until use in the experiment.
- 8.3.2 Reconstitute one of the provided 10 ng Lyophilized **Recombinant Human TNF standard**. Use one for each experiment. Prepare a stock 10,000 pg/mL **Human TNF standard** by reconstituting one tube of **Lyophilized Recombinant Human TNF standard as follows**:
- 8.3.2.1 Gently spin or tap the vial to collect all material at the bottom.
- 8.3.2.2 Add 1 mL of **Sample Diluent Buffer** to the vial.
- 8.3.2.3 Seal then mix gently and thoroughly.
- 8.3.2.4 Leave the vial to sit at ambient temperature for 10 minutes.
- 8.3.3 Prepare a set of seven serially diluted standards as follows:
- 8.3.3.1 Label tubes with numbers 1 – 8.
- 8.3.3.2 Add 300 μ L of **Sample Diluent Buffer** to Tube #'s 1 – 7.
- 8.3.3.3 Prepare a **1,000 pg/mL standard #1** in by adding 100 μ L of the 10,000 pg/mL reconstituted **Human TNF standard** to 900 μ L of **Sample Diluent Buffer** in Tube#1. Mix gently and thoroughly.
- 8.3.3.4 Prepare **standard #2** by adding 300 μ L of **1,000 pg/mL standard#1** from Tube #1 to Tube #2. Mix gently and thoroughly.
- 8.3.3.5 Prepare **standard #3** by adding 300 μ L of **standard #2** from Tube #2 to Tube #3. Mix gently and thoroughly.
- 8.3.3.6 Prepare further serial dilutions through Tube #7. Reference the table below as a guide for serial dilution scheme.
- 8.3.3.7 Tube #8 is a blank standard (only **Sample Diluent Buffer**), which should be included with every experiment.

Standard Number (Tube)	Sample To Dilute	Volume standard (μ L)	Volume Sample Diluent Buffer (μ L)	Total Volume (μ L)	Final Concentration
1	10,000 pg/mL of Human TNF standard	100	900	1,000	1,000 pg/mL
2	1,000 pg/mL	300	300	600	500 pg/mL
3	500 pg/mL	300	300	600	250 pg/mL
4	250 pg/mL	300	300	600	125 pg/mL
5	125 pg/mL	300	300	600	62.5 pg/mL
6	62.5 pg/mL	300	300	600	31.2 pg/mL
7	31.2 pg/mL	300	300	600	15.6 pg/mL
8	NA	0	300	300	0.0 (Blank)

8.4 1X Wash Buffer

- 8.4.1 Add 270 mL of ultra-pure water to a clean > 500 mL bottle or other vessel.
- 8.4.2 Add the entire 30 mL contents of the **10X Wash Buffer** bottle to the water.
- 8.4.3 Seal and mix gently by inversion. Avoid foaming or bubbles.
- 8.4.4 Store the **1X Wash Buffer** at room temperature until ready to use in the procedure. Store the prepared **1X Wash Buffer** at 4°C for no longer than 1 week. Do not freeze.

8.5 Microplate Preparation

- Micro-plates are provided ready to use and do not require rinsing or blocking.
- Unused well strips should be returned to the original packaging, sealed and stored at 4°C.
- Equilibrate microplates to ambient temperatures prior to opening to reduce potential condensation.

9. Sample Preparation

9.1 Sample Preparation and Storage

- Store samples to be assayed at 2-8°C for 24 hours prior being assayed.
- For long term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.
- Clear samples by centrifugation as follows:
 - **Cell culture supernatants** - Remove particulates by centrifugation, assay immediately or aliquot and store samples at -20°C.
 - **Serum** - Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature. Centrifuge at approximately 1,000 x g for 15 min. Analyze the serum immediately or aliquot and store samples at -20°C.
 - **Plasma** - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 min at 1,500 x g within 30 min of collection. Assay immediately or aliquot and store samples at -20°C.
 - **Saliva** - Collect saliva using a collection device without any protein binding or filtering capabilities such as a Salivette or aliquot and store samples at -20°C.

9.2 Sample Dilution

Target protein concentration must be estimated and appropriate sample dilution selected such that the final target protein concentration falls near the middle of the assay linear dynamic range.

- Prepare 150 µL sample for each replicate to be assayed.
- Dilute samples with Sample Diluent Buffer.
- Mix diluted samples gently and thoroughly.
- Pipetting less than 2 µL is not recommended for optimal assay accuracy.
- Refer to the following table for recommended sample dilution guidelines based on the dynamic range of this kit:

Estimated Sample Target Concentration		Dilution Level	Sample Volume For Two Replicates	Sample Diluent Buffer For Two Replicates
High Concentration	10-100 ng/mL	1:100	1 µL	99 µL
Medium Concentration	1-10 ng/mL	1:10	10 µL	90 µL
Low Concentration	15.6-1,000 pg/mL	1:2	50 µL	50 µL
Very Low Concentration	≤ 15.6 pg/mL	1:2 or No Dilution	-	-

10. Assay Procedure

- Equilibrate all reagents and materials to ambient room temperature prior to use in the procedure.
- Optimal results for intra- and inter-assay reproducibility will be obtained when performing all incubation steps at 37°C as indicated below.

- 10.1** Add 100 μ L of serially titrated standards, diluted samples or blank into wells of the pre-coated well plate. At least two replicates of each standard, sample or blank is recommended.
- 10.2** Cover the plate with the well plate lid and incubate at 37°C for 90 minutes.
- 10.3** Remove the plate lid and discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle.
- 10.4** Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.
- 10.5** Add 100 μ L of prepared **1X Biotinylated Anti-Human TNF Antibody to each well.**
- 10.6** Cover with the well-plate lid and incubate at 37°C for 60 minutes.
- 10.7** Wash plate 3 times with **1X Wash Buffer** as follows:
 - 10.7.1 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle.
 - 10.7.2 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.
 - 10.7.3 Add 300 μ L of **1X Wash Buffer** to each assay well.
 - 10.7.4 Incubate for 1 minute.
 - 10.7.5 Repeat steps 10.7.1 through 10.7.4 **two** more times.
- 10.8** Add 100 μ L of prepared **1X Avidin-Biotin-Peroxidase Complex (ABC)** into each well and incubate at 37°C for 30 minutes.
- 10.9** Wash plate 5 times with **1X Wash Buffer** as follows:
 - 10.9.1 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle.
 - 10.9.2 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.
 - 10.9.3 Add 300 μ L of 1X Wash Buffer to each assay well.
 - 10.9.4 Incubate for 1 minute.
 - 10.9.5 Repeat steps 10.9.1 through 10.9.4 **four** more times.
- 10.10** Add 90 μ L of **TMB Color Developing Agent** to each well and incubate at 37°C in the dark for 25-30 minutes. (NOTE: optimal incubation time must be determined by the user. Optimal development can be visualized by blue shading in the top four standard wells, while the remaining standards are still clear.)
- 10.11** Add 100 μ L of **TMP Stop Solution** to each well. Well color should change to yellow immediately.
- 10.12** Read the O.D. absorbance at 450 nm with a standard microplate reader within 30 minutes of stopping the reaction in step 10.11.

11. Calculation of Results

For analysis of the assay results, calculate the **Relative OD₄₅₀** for each test or standard well as follows:

$$(\text{Relative OD}_{450}) = (\text{Well OD}_{450}) - (\text{Mean Blank Well OD}_{450})$$

The standard curve is generated by plotting the mean replicate **Relative OD₄₅₀** of each standard serial dilution point vs. the respective standard concentration. The Human TNF concentration contained in the samples can be interpolated by using linear regression of each mean sample **Relative OD₄₅₀** against the standard curve. This is best achieved using curve fitting software.

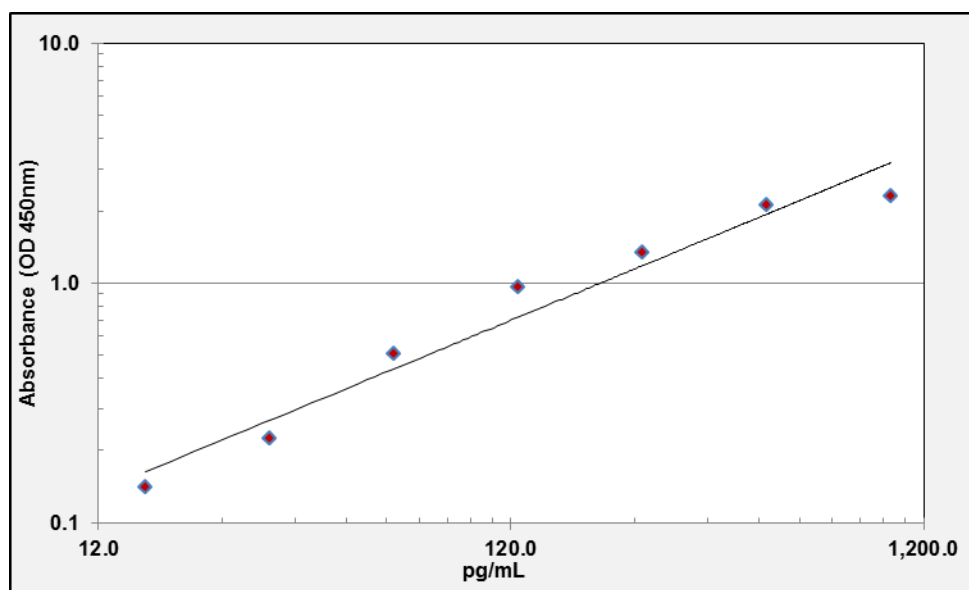
Note: if the samples measured were diluted, multiply the derived mean sample concentration by the dilution factor for a final sample concentration.

12. Typical Expected Data

12.1 Typical absorbance values. Expected absorbance for standards when TMB incubation is performed for 20 minutes at 37°C and measured at OD₄₅₀.

Standard Number	8	7	6	5	4	3	2	1
standard Concentration (pg/mL)	0	15.6	31.2	62.5	125	250	500	1,000
OD ₄₅₀	0.039	0.140	0.225	0.509	0.960	1.333	2.112	2.309

12.2 Typical standard curve. This standard curve is for demonstration purposes only. An assay specific standard curve should be performed with each assay.



12.3 General Specifications

General Specifications	
Range	15.6 pg/mL - 1,000 pg/mL (cell culture supernates) 7.8 pg/mL – 500 pg/mL (serum, plasma)
Sensitivity	< 1 pg/mL (Derived by linear regression of OD ₄₅₀ of the Mean Blank + 2xSD)
Specificity	Natural and recombinant Human TNF UniProt ID: P01375
Cross-Reactivity	No detectable cross-reactivity with other relevant proteins

12.4 Reproducibility

Sample ID	Intra-Assay			Inter-Assay		
	1	2	3	1	2	3
n =	16	16	16	24	24	24
Mean Measured Concentration (pg/mL)	93	327	608	102	319	613
Standard Deviation	5.1	15.4	31	7.65	15.3	35
Consistency (%CV)	5.5	4.7	5.1	7.5	4.8	5.7

13. Technical Resources

13.1 References

- 13.1.1 Brenner, D. A.; O'Hara, M.; Angel, P.; Chojkier, M.; Karin, M. Prolonged activation of JUN and collagenase genes by tumour necrosis factor-alpha. *Nature* 337: 661-663, 1989.
- 13.1.2 Nedwin, G. E.; Naylor, S. L.; Sakaguchi, A. Y.; Smith, D.; Jarrett-Nedwin, J.; Pennica, D.; Goeddel, D. V.; Gray, P. W. Human lymphotoxin and tumor necrosis factor genes: structure, homology and chromosomal localization. *Nucleic Acids Res.* 13: 6361-6373, 1985.
- 13.1.3 Broudy, V. C.; Kaushansky, K.; Segal, G. M.; Harlan, J. M.; Adamson, J. W. Tumor necrosis factor type alpha stimulates human endothelial cells to produce granulocyte/macrophage colony-stimulating factor. *Proc. Nat. Acad. Sci.* 83: 7467-7471, 1986.
- 13.1.4 Wang, A. M.; Creasey, A. A.; Ladner, M. B.; Lin, L. S.; Strickler, J.; Van Arsdell, J. N.; Yamamoto, R.; Mark, D. F. Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science* 228: 149-154, 1985.

13.2 Technical Support

USA

Aviva Systems Biology, Corp.
5754 Pacific Center Blvd, Suite 201
San Diego, CA 92121

Phone: 858-552-6979
Toll Free: 888-880-0001
Fax: 858-552-6975

Technical support: techsupport@avivasysbio.com

China

Beijing AVIVA Systems Biology
6th Floor, B Building, Kaichi Tower
#A-2 Jinfu Road.
Daxing Industrial Development Zone
Beijing, 102600, CHINA

Phone: (86)10-60214720
Fax: (86)10-60214722
E-mail: support@avivasysbio.com.cn

中国地址: 北京大兴工业开发区金辅路甲 2 号凯驰大厦 B 座 6 层 (102600)
电话: 010-60214720/21
传真: 010-60214722

产品售前咨询及销售: sales@avivasysbio.com.cn
售后及技术支持: support@avivasysbio.com.cn