

**TNF ELISA Kit Human)
(OKAA00027_96W)
Instructions for Use**

For the quantitative measurement of TNF in cell culture supernates, serum, and plasma

Variations between lots 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) : 96 Wells (OKAA00027_96W) is based on standard sandwich enzyme-linked immuno-sorbent assay technology. An antibody specific for TNF has been pre-coated onto a 96-well plate (12 x 8 Well Strips). Standards or test samples are added to the wells, incubated and washed. A biotinylated detector antibody specific for TNF is added, incubated and followed by washing. Avidin-Peroxidase Conjugate is then added, incubated and unbound conjugate is washed away. An enzymatic reaction is produced through the addition of TMB substrate which is catalyzed by HRP generating a blue color product that changes to yellow after adding acidic stop solution. The density of yellow coloration read by absorbance at 450 nm is quantitatively proportional to the amount of sample TNF captured in the well.

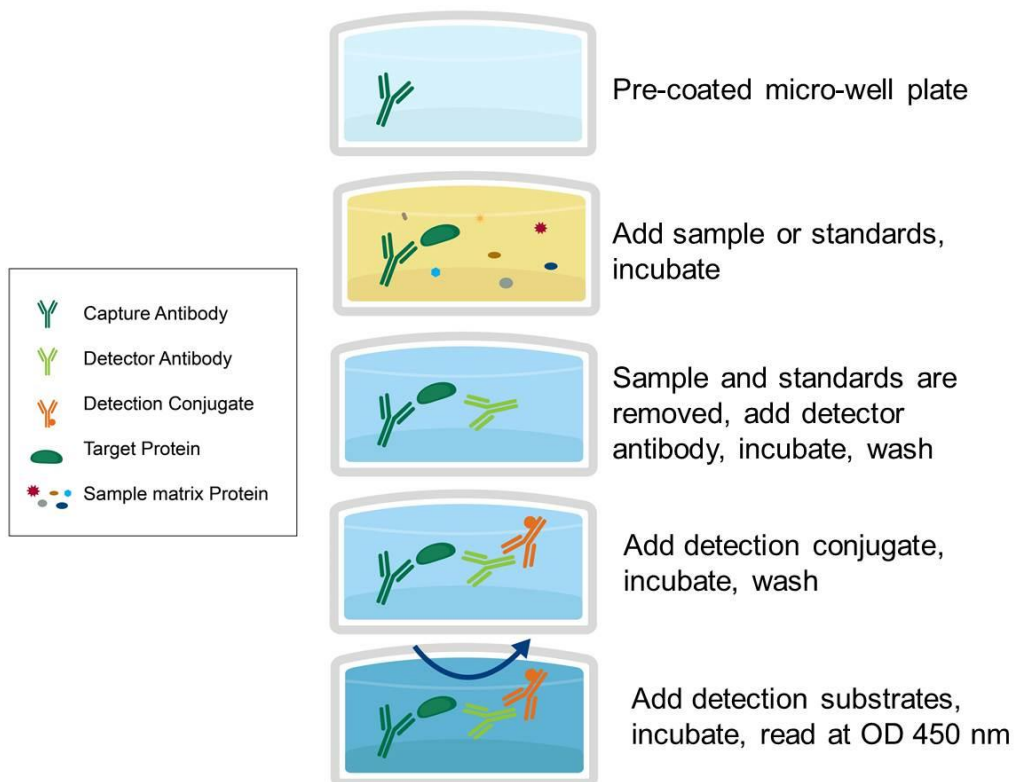
Target Background

Cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFR2. It is mainly secreted by macrophages and can induce cell death of certain tumor cell lines. It is potent pyrogen causing fever by direct action or by stimulation of interleukin-1 secretion and is implicated in the induction of cachexia. Under certain conditions it can stimulate cell proliferation and induce cell differentiation. Impairs regulatory T-cells (Treg) function in individuals with rheumatoid arthritis via FOXP3 dephosphorylation. Upregulates the expression of protein phosphatase 1 (PP1), which dephosphorylates the key 'Ser-418' residue of FOXP3, thereby inactivating FOXP3 and rendering Treg cells functionally defective. Key mediator of cell death in the anticancer action of BCG-stimulated neutrophils in combination with DIABLO/SMAC mimetic in the RT4v6 bladder cancer cell line.

General Specifications

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LOD	7 pg/ml (Derived by linear regression of OD ₄₅₀ of the Mean Blank + 2xSD)
Specificity	Human Tumor necrosis factor <u>UniProt ID</u> : P01375 <u>GeneID</u> : 7124 <u>Target Alias</u> : Cachectin, DIF, TNFA, TNF-a, TNF-alpha, TNFSF2, Tumor necrosis factor, Tumor necrosis factor ligand superfamily member 2
Cross-Reactivity	No detectable cross-reactivity with other relevant proteins

2. Assay Summary



3. Storage and Stability

- Store entire kit at 4°C. Do not use past expiration date.

4. Kit Components

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

Description	Quantity	Storage Conditions
TNF Microplate	96 Wells (12 x 8 Well strips)	Store at 4°C Do not use past expiration date
TNF Lyophilized Standard	2 x 4000 pg	
100X Biotinylated TNF Detector Antibody	2 x 60 µL	
100X Avidin-HRP Conjugate	2 x 60 µL	
Sample/Standard Diluent	1 x 16 mL	
Detector Antibody Diluent	1 x 12 mL	
Conjugate Diluent	1 x 12 mL	
20X Wash Buffer	1 x 25 mL	
Stop Solution	1 x 12 mL	
TMB Substrate	1 x 12 mL	

5. 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).
- Kit cannot be used beyond the expiration date on the label.

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 μ L 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.
- 37°C Incubator (optional)

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.
- Prior to using the kit, briefly spin component tubes to collect all reagents 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 for inter- and 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 bilirubin, precipitates or fibrin strands or are hemolytic or lipemic might cause inaccurate results due to interfering factors.
- TMB Substrate is easily contaminated and should be colorless or light blue until added to plate. Handle carefully and protect from light.

8. Reagent Preparation

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

8.1 Human TNF Assay Standards

- 8.1.1 Prepare the standards no greater than 2 hours prior to performing experiment. Standards should be held on ice until use in the experiment.
- 8.1.2 Reconstitute one vial of the provided 4 ng **Lyophilized Standard** for each experiment. Prepare a stock **4,000 pg/mL Standard** by reconstituting one tube of 4 ng **Lyophilized Standard** as follows:
- 8.1.2.1 Gently spin or tap the vial at 6,000 – 10,000 rpm for 30 seconds to collect all material at the bottom.
- 8.1.2.2 Add 1.0 mL of **Standard/Sample Diluent** to the vial.
- 8.1.2.3 Seal the vial then mix gently and thoroughly.
- 8.1.2.4 Leave the vial at ambient temperature for 15 minutes.
- 8.1.3 Prepare a set of serially diluted standards as follows:
- 8.1.3.1 Label tubes with numbers 1 – 8.
- 8.1.3.2 Add 300 μ L of **Standard Diluent** to Tube #'s 2 – 8.
- 8.1.3.3 Prepare **Standard #1** by adding 250 μ L of **4,000 pg/mL Standard** to 750 μ L of **Standard/Sample Diluent** in Tube #1. Mix gently and thoroughly.
- 8.1.3.4 Prepare **Standard #2** by adding 300 μ L of **Standard #1** (Tube #1) to Tube #2. Mix gently and thoroughly.
- 8.1.3.5 Prepare **Standard #3** by adding 300 μ L of **Standard #2** from Tube #2 to Tube #3. Mix gently and thoroughly.
- 8.1.3.6 Prepare further serial dilutions through Tube #7. Reference the table below as a guide for serial dilution scheme.
- 8.1.3.7 Tube #8 is a blank standard (only **Standard Diluent**), which should be included with every experiment.

Standard Number (Tube)	Standard To Dilute	Volume Standard to Dilute (μ L)	Volume Standard Diluent Buffer (μ L)	Total Volume (μ L)	Final Concentration
1	Reconstituted 4,000 pg/mL Standard	250	750	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	31.25 pg/mL
7	31.25 pg/mL	300	300	600	15.63 pg/mL
8	NA	0	300	300	0.0 (Blank)

8.2 **1X Biotinylated TNF Detector Antibody**

- 8.2.1 Prepare the **1X Biotinylated TNF Detector Antibody** immediately prior to use by diluting the **100X Biotinylated TNF Detector Antibody** 1:100 with **Detector Antibody Diluent**.
- 8.2.2 For each well strip to be used in the experiment (8-wells) prepare 1,000 μL by adding 10 μL of **100X Biotinylated TNF Detector Antibody** to 990 μL **Detector Antibody Diluent**.
- 8.2.3 Mix thoroughly and gently. Hold no longer than 2 hours prior to using in procedure. Do not store at 1X concentration for future use.

8.3 **1X HRP-Avidin Conjugate**

- 8.3.1 Prepare the **1X Avidin-HRP Conjugate** immediately prior to use by diluting the **100X Avidin-HRP Conjugate** 1:100 with **Conjugate Diluent**.
- 8.3.2 For each well strip to be used in the experiment (8-wells) prepare 1,000 μL by adding 10 μL of **100X Avidin-HRP Conjugate** to 990 μL **Conjugate Diluent**.
- 8.3.3 Mix thoroughly and gently. Hold no longer than 2 hours prior to using in procedure. Do not store at 1X concentration for future use.

8.4 **1X Wash Buffer**

- 8.4.1 If crystals have formed in the **20X Wash Buffer** concentrate, equilibrate to room temperature and mix gently until crystals have completely dissolved.
- 8.4.2 Add 10 mL of the **20X Wash Buffer** bottle to 190 mL of ultra-pure water to a clean > 500 mL bottle or other vessel.
- 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 °4C.
- 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 4°C for 24 hours prior being assayed.
- For long term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.
- Samples not indicated in the manual must be tested to determine if the kit is valid.
- Prepare samples as follows:
 - **Serum** - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1,000 x g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.
 - **Plasma** - Collect plasma using EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1,000 x g at 4°C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.
 - **Tissue Homogenates** – Rinse 100 mg of tissue with 1X PBS, then homogenize in 20 ml of 1X PBS and store overnight at -20°C. Perform two freeze-thaw cycles to lyse the cell membranes, then centrifuge the homogenates for 5 minutes at 5,000 x g, 4°C. Remove the supernatant and assay immediately. Alternatively, aliquot and store samples at -20°C or -80°C. Centrifuge the sample again after thawing before the assay. Avoid repeated freeze-thaw cycles.
 - **Cell culture supernatants and other biological fluids** – Remove particulates by centrifugation and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze/thaw cycles.

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. Samples exhibiting saturation should be further diluted.

- Dilute samples using **Sample Diluent**.
- Mix diluted samples gently and thoroughly.
- Pipetting less than 2 µL is not recommended for optimal assay accuracy.
- Note: The normal mouse serum or plasma samples are suggested to make a 1:2 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 incubation steps at 37°C as indicated below.

- 10.1** Determine the required number of wells and return any remaining unused wells and desiccant to the pouch.
- 10.2** Add 100 µL of serially titrated standards, diluted samples or blank into wells of the **TNF Microplate**. At least two replicates of each standard, sample or blank is recommended.
- 10.3** Cover the plate with the well plate lid and incubate at 37°C for 1.5 hours.
- 10.4** Remove the plate lid and discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.
- 10.5** 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.
- 10.6** Wash plate 4 times with **1X Wash Buffer** as follows:
 - 10.6.1 Add 300 µL of **1X Wash Buffer** to each assay well.
 - 10.6.2 Soak for 5 seconds.
 - 10.6.3 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle.
 - 10.6.4 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.
 - 10.6.5 Repeat steps 10.6.1 through 10.6.4 **three** more times.
- 10.7** Add 100 µL of prepared **1X Biotinylated TNF Detector Antibody** to each well.
- 10.8** Cover with the well-plate lid and incubate at 37°C for 60 minutes.
- 10.9** Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.
- 10.10** 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.
- 10.11** Wash plate as in Step 10.6.
- 10.12** Add 100 µL of prepared **1X Avidin-HRP Conjugate** into each well and incubate at 37°C for 30 minutes.
- 10.13** Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.
- 10.14** 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.
- 10.15** Wash plate as in Step 10.6.
- 10.16** Add 100 µL of **TMB Substrate** to each well and incubate at 37°C in the dark for 10 - 20 minutes. Wells should change to gradations of blue. If the color is too deep, reduce the incubation time.
(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.)
- 10.17** Add 100 µL of **Stop Solution** to each well. Well color should change to yellow immediately. Add the **Stop Solution** in the same well order as done for the **TMB Substrate**.
- 10.18** Read the O.D. absorbance at 450 nm with a standard microplate reader within 5 minutes of stopping the reaction in step 10.17. If wavelength correction is available, set to 630 nm or 610-650 nm.

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 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 wavelength correction readings were available, subtract the readings at 540 nm or 570 nm from the readings at 450 nm. This may provide greater reading accuracy.

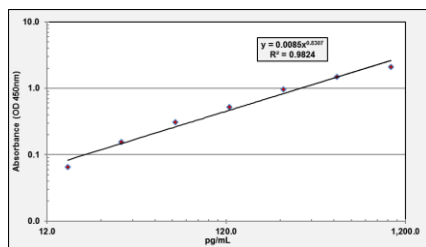
Note: if the samples measured were diluted, multiply the derived mean sample concentration by the dilution factor for a final sample concentration.

12. Typical Expected Data

12.1 Reproducibility

Coefficient of variation of both intra-assay and inter-assay are less than 10%.

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



pg/mL	Absorbance		Mean Absorbance	Blank Subtracted
	Rep 1	Rep 2		
1,000	2.200	2.217	2.209	2.119
500	1.577	1.564	1.571	1.481
250	1.059	1.042	1.051	0.961
125	0.618	0.609	0.614	0.524
62.5	0.391	0.401	0.396	0.307
31.25	0.248	0.239	0.244	0.154
15.63	0.153	0.156	0.155	0.065
Blank	0.086	0.093	0.090	NA

12.3 Specificity

This assay recognizes both natural and recombinant human TNF. The factors listed below were prepared at 70 ng/mL in Standard /Sample Diluent and assayed for cross-reactivity and no significant cross-reactivity or interference was observed.

Recombinant human	Recombinant mouse	Recombinant pig
TNFb	TNFa	TNFa
GM-CSF	TNFa (truncated)	
sTNF RI	sTNF RI	
sTNF RII	sTNF RII	

13. Technical Resources

Technical Support:

For optimal service please be prepared to supply the lot number of the kit used.

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