Monkey TNF alpha ELISA Kit

Enzyme Immunoassay for the quantification of Monkey TNF alpha in Monkey serum, plasma, cell culture supernatants

Catalog number: ARG81999

For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

This gene encodes a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. This cytokine is mainly secreted by macrophages. 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. This cytokine has been implicated in a variety of diseases, including autoimmune diseases, insulin resistance, and cancer. Knockout studies in mice also suggested the neuroprotective function of this cytokine. [provided by RefSeq, Jul 2008]

Cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. 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.

The TNF intracellular domain (ICD) form induces IL12 production in dendritic cells. [UniProt]

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for TNF alpha is used to coat onto a 96well microtiter plate. Standards or samples are pipetted into the wells and any TNF alpha present is bound by the immobilized antibody. After washing away any unbound substances, a biotin-conjugated antibody specific for TNF alpha is added to each well and incubate. Following a washing to remove unbound substances, streptavidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After washing away any unbound antibody-enzyme reagent, a substrate solution (TMB) is added to the wells and color develops in proportion to the amount of TNF alpha bound in the initial step. The color development is stopped by the addition of acid and the intensity of the color is measured at a wavelength of 450nm ±2nm. The concentration of TNF alpha in the sample is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8°C. Store HRP-Streptavidin concentrate at ≤-20°C in dark. Use the kit before expiration date.

Component	**Quantity**	**Storage information**
ELISA microplate	8 plates	Room temperature
Standard	5 X 0.64 ng/vial (Lyophilized)	4°C
Coating antibody	1 vials (lyophilized)	4°C
Antibody conjugate concentrate	1 vials (lyophilized)	4°C
HRP-Streptavidin concentrate	1 vials (lyophilized)	≤-20°C (Protect from light)
10% BSA stock solution	2 x 12 ml	4°C
Tween-20	5 ml (Ready to use)	RT
Cytokine stabilization buffer (CSB)	5 ml (Ready to use)	4°C
TMB substrate	2 X 30 ml (Ready to use)	4°C (Protect from light)
STOP solution	2 X 30 ml (Ready to use)	4°C
Plate sealer	10 strips	Room temperature

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 370, 450 and 655 nm
- Pipettes and pipette tips
- Sterile deionized or distilled water
- 37°C oven or incubator
- PBS (pH 7.4; ingredients: $Na_2HPO_4 \cdot 2H2O$, KH_2PO_4, NaCl and distilled water). Alternatively, use commercially available liquid PBS (pH 7.4)
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C at all times. Store HRP-Streptavidin concentrate at ≤-20°C in dark.
- To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, a pilot experiment using standards and a small number of samples is recommended.
- The TMB Color developing agent should be colorless and transparent before using. Store the TMB solution at 4°C and avoid exposure to light, heat and contamination with metal ions or peroxidase.
- Opened BSA stock solution and Cytokine stabilization buffer can be stored at 4°C for up to 6 months when kept sterile.
- Ensure complete reconstitution and dilution of reagents prior to use.
- All reagents should be mixed by gentle inversion or swirling prior to use.

Do not induce foaming.

- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- Do not let strips dry, as this will inactivate active components in wells.
- It is highly recommended that the standards, samples and controls be assayed in duplicates.
- Change pipette tips between the addition of different reagent or samples.
- Avoid using reagents from different batches.
- In order to avoid marginal effect of plate incubation due to temperature difference (reaction may be stronger in the marginal wells), it is suggested that the 1X HRP-Streptavidin Solution and TMB substrate be pre-warmed at RT for 10 min before use.
- Samples contain azide cannot be assayed.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernatants - Remove particulates by centrifugation for 10 min at 1500 x g at RT and aliquot & store samples at -20°C up to 1 month or-80°C up to 6 months. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Collect serum and assay immediately or aliquot & store samples at -20°C up to 1 month or -80°C up to 6 months. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g. within 30 minutes of collection. Collect the supernatants and assay immediately or aliquot and store samples at -20°C up to 1 month or-80°C up to 6 months. Avoid repeated freeze-thaw cycles.

Note:

1. When measuring cytokines in serum or plasma, add 1/20 volume of Cytokine stabilization buffer (CSB; ready-to-use) to the pure serum or plasma sample (CSB is not required for other samples such as cell culture supernatant) before further dilution in Dilution buffer. CSB inhibits the degradation of cytokines.
2. Do not heat serum or plasma samples. Prior to assay, frozen samples should be completely thawed and mixed well.
3. Specimen collection from humans and non-human primates should be carried out in accordance with NCCLS document M29-T2, "Protection of laboratory workers from infectious diseases transmitted by blood and tissue".

REAGENT PREPARATION

- **PBS:**

  PB stock: dissolve 96.0 g of $Na_2HPO_4 \cdot 2H_2O$ plus 17.5 g of KH_2PO_4 in 800 ml of distilled water, adjust pH to 7.4 and then add distilled water to 1L. Solution should be filter sterilized (0.2 µm) or autoclaved. Store solution at RT (stable for at least 6 months when kept sterile).

  PBS: add 10 ml of the PB stock and 8.8 g of NaCl to 1 L distilled water. It is strongly recommended to prepare PBS freshly each day. Alternatively, when PBS is prepared in advance, the solution should be filter sterilized (0.2 µm) or autoclaved.

- **1X Wash Buffer:** PBS containing 0.05% Tween-20 (add 0.5 ml of Tween-20 to 1 L PBS). The volume is depending on the washing procedure (manual or automatic washing).

- **Blocking buffer**: PBS containing 1% BSA. For one ELISA plate: mix 2 ml BSA stock solution (10%) gently but thoroughly with 18 ml PBS.

- **Dilution buffer:** PBS containing 0.5% BSA and 0.05% Tween-20. You can prepare this buffer at once for 5 ELISA plates by making at least 250 ml under sterile conditions. Add 12.5 ml of BSA stock solution (10%) and 125 µl of Tween-20 to 250 ml PBS, mix gently and store at 4°C. This solution will be stable for at least one month when kept sterile.

  For one ELISA plate, 20 ml of Dilution buffer is needed for detection and conjugate solutions, and at least 20 ml for standards and samples (this volume will depend on the number of sample dilutions).

- **Coating Antibody**: The lyophilized Coating Antibody could be stored at 4°C until the expiry date. Reconstitute the Coating Antibody with 250 µl of sterile distilled water, mix the solution gently for 15-30 sec and keep the antibody in the vail for 5 min at RT to completely dissolve. Avoid vigorous shaking. After reconstitution, the antibodies are stable for at least 12 months at 4°C when kept sterile. However, we recommended aliquot and store the antibody stock at-20°C (stable for at least one years). Avoid repeated freeze-thaw cycles.

  For one ELISA plate: Gently add 50 µl of reconstituted Coating Antibody in 5 ml of PBS, mix thoroughly before use.

  Note: Do not use commercially available PBS tablets for the preparation of the coating solution (the filler in the tablets interferes with the coating process).
- **1X Antibody conjugate**: The lyophilized biotin-conjugated antibody could be stored at 4°C until the expiry date. Reconstitute the Antibody conjugate concentrate with 500 µl of sterile distilled water, mix the solution gently for 15-30 sec and keep the antibody in the vail for 5 min at RT to completely dissolve. Avoid vigorous shaking. After reconstitution, the antibodies are stable for at least 12 months at 4°C when kept sterile. However, we recommended aliquot and store the antibody stock at ≤ -20°C (stable for at least one years). Avoid repeated freeze-thaw cycles.

  For one ELISA plate: Gently add 100 µl of reconstituted Antibody conjugate in 10 ml of Dilution buffer, mix thoroughly before use.

- **1X HRP-Streptavidin Solution**: The lyophilized HRP-Streptavidin Solution could be stored at ≤-20°C in dark until the expiry date. Reconstitute the HRP-Streptavidin Solution with 500 µl of sterile distilled water, mix the solution gently for 15-30 sec and keep the antibody in the vail for 5 min at 4°C in dark to completely dissolve. Avoid vigorous shaking. After reconstitution, the antibodies are stable for at least 2 months at 4°C in dark when kept sterile. However, we recommended aliquot and store the antibody stock at ≤ -20°C in dark (stable for at least one years). Avoid repeated freeze-thaw cycles.

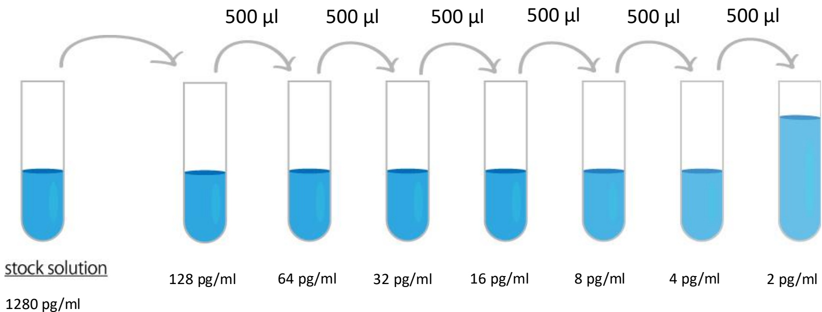
  For one ELISA plate: Gently add 100 µl of reconstituted HRP-Streptavidin Solution in 10 ml of Dilution buffer, mix thoroughly before use.
- **Sample**: When measuring cytokines in serum or plasma, add 1/20 volume of Cytokine stabilization buffer; (CSB, ready-to-use) to the pure serum or plasma sample (CSB is not required for other samples such as cell culture supernatant) before further dilution in Dilution buffer. CSB inhibits the degradation of cytokines.

  Dilute samples at least 1:1 with Dilution buffer before assay. If the initial assay found samples contain TNF alpha higher than the highest standard, the samples can be diluted with Standard/Sample diluent and then re-assay the samples. For the calculation of the concentrations this dilution factor has to be taken into account. The sample must be well mixed with the diluents buffer before assay.

  (It is recommended to do pre-test to determine the suitable dilution factor).

- **Standards**: Standard solution should be prepared within one hour prior to the experiment. Reconstitute the standard with 0.5 ml sterile distilled water to yield a stock concentration of 1280 pg/ml. Mix the solution gently for approximately 15 seconds and allow the stock standard to sit for at least 5 minutes at RT with gentle agitation to make sure the standard is dissolved completely before making serial dilutions. Avoid vigorous shaking. Keep the reconstituted standard on ice before use. The dilution buffer serves as zero standard (0 pg/ml), and the rest of the standard serial dilution can be diluted with dilution buffer as according to the suggested concentration below: 128 pg/ml, 64 pg/ml, 32 pg/ml, 16 pg/ml, 8 pg/ml, 4 pg/ml, 2 pg/ml.

  Note: The reconstituted and diluted standard solutions should be used within one hour.



Dilute TNF alpha standard as according to the table below:

Standard	TNF alpha Conc. (pg/ml)	µl of dilution buffer	µl of standard
S7	128 pg/ml	900	100 (1280 pg/ml Stock)
S6	64 pg/ml	500	500 (S7)
S5	32 pg/ml	500	500 (S6)
S4	16 pg/ml	500	500 (S5)
S3	8 pg/ml	500	500 (S4)
S2	4 pg/ml	500	500 (S3)
S1	2 pg/ml	500	500 (S2)
S0	0	500	0

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT) before use. When diluting samples and reagents, they must be mixed completely and evenly. Standard TNF alpha detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of TNF alpha amount in samples. Standards, samples and controls should be assayed in duplicates or triplicate.

1. Add 50 µl of diluted coating antibody solution to each well of the ELISA plate and fill up to 100 µl with PBS. Seal the plate to prevent evaporation.
2. Incubate overnight at 4°C (or alternatively 2 hours at 37°C).
3. Aspirate each well and wash (without touching the bottom), repeating the process five times for a total six washes. Wash by filling each well with 1X Wash Buffer (300 µl) using a squirt bottle, manifold dispenser, or autowasher, keep the wash buffer in the wells for 1 min before remove. Complete removal of liquid at each is essential to good performance. After

the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels. DO NOT let the wells completely dry at any time. If too much bubbles when using a squirt bottle are occurred, replace wash buffer by PBS for the last washing step.

4. Add 200 µl of Blocking buffer to each well. Seal the plate and incubate for 1 hour at 37°C.
5. Remove the Blocking buffer (do not wash the wells).
6. Add 100 µl of diluted standard/blank/samples to each well.
7. Seal the plate and incubate for 2 hours at 37°C (or alternatively overnight at 4°C).
8. Remove standards/samples and wash the wells as step 3.
9. Add 100 µl of 1X Antibody conjugate to each well. Seal the plate and incubate for 1 hour at 37°C.
10. Remove detection antibody solution and wash the wells as step 3.
11. Add 100 µl of 1X HRP-Streptavidin solution to each well. Seal the plate and incubate for 1 hour at 37°C.
12. Remove 1X HRP-Streptavidin solution and wash the wells as step 3.
13. Add 100 µl of TMB substrate to each well. Incubate for 20 minutes at RT in dark. (Note: The incubation time is for reference only, the optimal incubation time should be determined by end user. And the shades of blue color can be seen in the wells with the four most concentrated TNF alpha standard solutions; the other wells show no obvious color. The substrate produces a soluble blue end product that can be read at 370 or 655 nm.)
14. Add 100 µl of Stop Solution to each well. The color of the solution should change from blue to yellow. Gently tap the plate to ensure thorough

mixing.

15. Read the OD with a microplate reader at 450nm immediately. It is recommended read the absorbance within 30 minutes after adding the stop solution.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.

2. Using linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

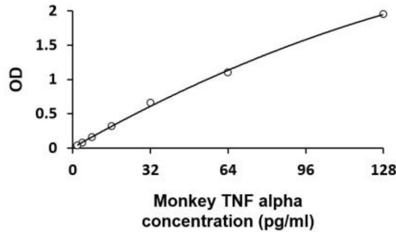
3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

5. If the samples have been diluted, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure as described above.

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

The minimum detectable dose (MDD) of Monkey TNF alpha standard ranged from 2- 128 pg/ml. The mean MDD was 2 pg/ml.

Specificity

This assay recognizes natural and recombinant Monkey TNF alpha (Rhesus macaque, Cynomolgus monkey, Pig-tailed macaque, Barbary macaque, Baboon, African green monkey, Marmoset). No significant cross-reactivity or interference with the factors below was observed:

sTNF receptors (RI/RII) at a 1000-fold molar excess