

Human TNF- α ELISA Kit

to determine Human TNF- α in Serum, Blood Plasma, Saliva, Urine, Tissue
Liquid Samples or related Biological Solutions.

INSTRUCTION MANUAL FOR ELISA KIT No: LT28100EAYQ



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INTENDED USE

This kit is used to determine the Human TNF- α in the sample of serum, blood plasma, saliva, urine, tissue liquid samples or related biological solutions. For in vitro use only.

MANUAL VERSION 1.02 B

BACKGROUND

TNF- α is synthesized as a 26 kDa, type II transmembrane protein that is 233 amino acids in length. It contains a 30 amino acid (aa) cytoplasmic domain, a 26 aa transmembrane segment, and a 177 aa extracellular region. TNF- α is assembled intracellularly to form a transmembrane, non-covalently-linked homotrimeric protein. The 157 aa residue soluble form of TNF- α (sTNF- α) is released from the C-terminus of the transmembrane protein through the activity of TNF- α -converting enzyme (TACE), a membrane - bound disintegrin metalloproteinase. Rat cells known to express TNF- α include B cells, colonic columnar epithelial cells, NK and CD3 CD56 hepatic natural T cells, macrophages, monocytes and monocyte-derived dendritic cells, CD4 and CD8 T cells, mast cells, neutrophils, keratinocytes, plasma cells, and adipocytes.

PRINCIPLE

Human TNF- α ELISA Kit employs a two-site sandwich ELISA to quantitate TNF- α in samples. An antibody specific for TNF- α has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TNF- α present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for TNF- α is added to the wells. After washing, Streptavidin-HRP conjugate is added to the wells. Following a wash to remove any unbound streptavidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TNF- α bound in the initial step. The color development is stopped and the intensity of the color is measured.

ASSAY RESTRICTIONS

- Do not mix or substitute reagents with those from other lots or sources.
- It is important that the calibrator diluent selected for the standard curve be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, dilute the samples with the appropriate calibrator diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the ELISA Immunoassay, the possibility of interference cannot be excluded.

MATERIALS SUPPLIED IN THIS KIT

Human TNF- α microplate: 96 well polystyrene microplates (12 strips of 8 wells) coated with the antibody specific for Human TNF- α .

Human TNF- α standard: Human IFN- γ in a buffered protein base; lyophilized.

Sample diluent: Diluent solution for reconstituting standard and samples. 5X liquid.

Human TNF- α detect antibody: Biotin-conjugated Human TNF- α detection antibody. 100X liquid.

Streptavidin-HRP: 100X liquid.

Assay buffer: Diluent solution for detection antibodies and Streptavidin-HRP. 5X liquid.

HRP substrate: TMB (Tetramethyl-benzidine) solution.

Stop solution: 2N sulfuric acid.

Wash buffer: PBS with 1% Tween-20; 20X liquid.

Plate covers.

MATERIALS REQUIRED BUT NOT SUPPLIED

1. 37 °C incubator
2. Microplate reader
3. Precision pipettes and tips
4. Distilled water
5. Disposable tubes for sample dilution
6. Absorbent paper

STORAGE CONDITIONS

The unopened kit should be stored at 2-8°C for 1 year. Immediately after use remaining reagents should be returned to cold storage at 4°C. Recommended storage instruction for opened/reconstituted kit components are listed below.

Kit components	Storage conditions for opened components
Human TNF- α microplate	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.
Human TNF- α standard	Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer. Avoid repeated freeze-thaw cycles.
Human TNF- α detect antibody, Streptavidin-HRP, Sample Diluent, Assay Buffer, Substrate, Stop Solution, Wash buffer	May be stored for up to 1 month at 2-8 °C.

PRECAUTIONS

Reagents provided in this kit may be harmful if ingested, inhaled or absorbed through the skin. Please carefully review the MSDS for each reagent before conducting the experiment.

Stop Solution contains 2 N Sulfuric Acid (H₂SO₄) and is an extremely corrosive agent. Please wear proper eye, hand and face protection when handling this

material. When the experiment is finished, be sure to rinse the plate with copious amounts of running water to dilute the Stop Solution prior to disposing the plate.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate solution should remain colourless until added to the plate. Keep Substrate solution protected from light. Substrate solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate solution.

PRECISION

Intra-assay Precision (Precision within an assay): Four samples of known concentration were tested twenty times on one plate to assess intra-assay precision. The CV (%) <10%.

Inter-assay Precision (Precision between assays): Three samples of known

concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components. The CV (%) <10%.

Recovery: The recovery of Human TNF- α spiked to different levels in samples throughout the range of the assay in various matrices was evaluated. The recovery ranged from 98% to 116% with an overall mean recovery of 106%.

Sensitivity: The minimum detectable dose (MDD) of Human TNF- α is typically less than 4 pg/ml. The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

Specificity: Human TNF- α ELISA Kit can be used to measure natural and recombinant Human TNF- α . Human TNF- α ELISA Kit has high sensitivity and excellent specificity for detection of Human TNF- α . No significant cross-reactivity or interference between Human TNF- α and analogues was observed.

SAMPLE COLLECTION & STORAGE

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 and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum

and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Plasma- Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens. Samples should be aliquoted and must be stored at -20°C to avoid loss of bioactive TNF- α . If samples are to be used within 24 hours, they may be stored at 2 to 8 $^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

REAGENT PREPARATION

Bring all reagents to room temperature before use. If crystals have formed in the Buffer Concentrates, warm them gently until they completely dissolved.

Wash buffer: Pour entire contents (50 ml) of the Wash buffer (20 \times) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle and store at 2 to 25 $^{\circ}\text{C}$. Please note that Wash buffer (1X) is stable for 30 days.

Sample Diluent and Assay buffer: Pour the entire contents (7 ml) of the Diluent (5X) into a clean 100 ml graduated cylinder. Bring to final volume of 35 ml with distilled water. Mix gently to avoid foaming. Store at 2 to 8 $^{\circ}\text{C}$. Please note that the Diluent (1X) is stable for 30 days.

HRP substrate: The reagents should be ready within 15 minutes of use. Protect from light. 100 μl of the solution is required per well.

Standard: In this kit two lyophilized vials have been provided. You should

reconstitute only one vial at a time and use the unopened vial later.

Reconstitute one Human TNF- α standard vial in 1ml of Sample Diluent for a concentration of 500 pg/ml. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Pipette 500 μ l of Sample Diluent into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Standard serves as the high standard (500 pg/ml). Sample Diluent serves as the zero standard (0 pg/ml).

Human TNF- α detect antibody: Mix well prior to making dilutions. Make a 1:100 dilution of the concentrated detect antibody solution with Assay buffer in a clean plastic tube as needed according to the standards and samples. Detect antibody should be used within 30 minutes after dilution.

Streptavidin-HRP - Mix well prior to making dilutions. Make a 1:100 dilution of the concentrated Streptavidin-HRP with Assay buffer in a clean plastic tube as needed according to the standards and samples. Streptavidin-HRP should be used within 30 minutes after dilution.

Sample dilution - If your samples need to be diluted, Sample diluent is used for dilution of serum/plasma samples, while cell culture medium is used for dilution of cell culture supernates.

ASSAY PROCEDURE

1. Dilution of standard solutions: This kit contains a standard of known concentration, which could be diluted in small tubes by the end-user by following the instruction in the table below:

250 pg/ml	Standard No.6	500µl Original Standard + 500µl Standard diluents
125 pg/ml	Standard No.5	500µl Standard No.5 + 500µl Standard diluents
62.5 pg/ml	Standard No.4	500µl Standard No.4 + 500µl Standard diluent
31.25 pg/ml	Standard No.3	500µl Standard No.3 + 500µl Standard diluent
15.6 pg/ml	Standard No.2	500µl Standard No.2 + 500µl Standard diluent
7.8 pg/ml	Standard No.1	500µl Standard No.1 + 500µl Standard diluent



Prepare all reagents and working standards as directed in the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µl of diluted standard and sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided for a record of standards and samples assayed.
4. Aspirate each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash buffer (250 µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or automatic washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Add 100 µl of diluted Human TNF- α detection antibody to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.
6. Repeat the aspiration/wash as in step 4.
7. Add 100 µl of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 30 minutes at room temperature. Avoid placing the plate in direct light.
8. Repeat the aspiration/wash process for five times as in step 4.

9. Add 100 μ l of HRP substrate solution to each well. Incubate for 15 minutes at room temperature. Protect from light.
10. Add 50 μ l of Stop solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set reference filter to 540 nm / 570 nm / 620 nm. If wavelength correction is not available, subtract readings at 540nm/570nm/620nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at only 450 nm without correction may be higher and less accurate.

PROTOCOL SUMMARY

Prepare reagents, samples and standards.



Add 100 μ l samples & standards in respective wells and incubate for 2 hours at RT.



Wash the plate three times. Add 100 μ l of detect antibody and incubate for 1 hour at RT.



Wash the plate three times. Add 100 μ l of diluted streptavidin-HRP to each well, incubate for 30 minutes at RT, and wash plate five times.



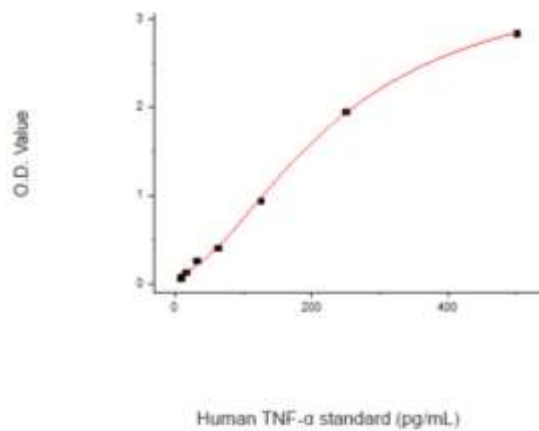
Add 100 μ l substrate solution and incubate for 10-15 minutes at RT in Dark.



Add 50 μ l stop solution to each well, measure O.D. at 450nm within 30 min.



Plot and Calculate.



Standard TNF- α (pg/mL)	Optical Density (450nm)	Average	Corrected
0	0.024 0.024	0.024	—
7.8	0.093 0.093	0.093	0.069
15.6	0.155 0.160	0.158	0.134
31.3	0.258 0.318	0.288	0.264
62.5	0.404 0.459	0.432	0.408
125	0.905 1.034	0.970	0.946
250	1.910 2.039	1.975	1.951
500	2.728 3.012	2.870	2.846

THE CHART SHOWN HERE IS FOR REFERENCE PURPOSES ONLY AND THE ACTUAL PERFORMANCE MAY VARY.

CALCULATION

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.). Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the concentration on the y-axis against the mean absorbance for each standard on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the Human TNF- α concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

ASSAY RANGE : 7.8 pg/ml - 500 pg/ml.

PACKAGE SIZE : 96Tests.

SENSITIVITY : <4 pg/ml.

Linearity: To assess linearity of the assay, samples containing and/or spiked with high concentrations of Human TNF- α were diluted with the appropriate calibrator diluent to produce samples with values within the dynamic range of the assay. Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99.

VALIDITY & STORAGE: 12 months (at 2-8°C, unopened).