

## Human TNF-a ELISA Kit

**Catalog No.:** CKH111

**Lot No.:**

**Size:** 5 x 96 tests

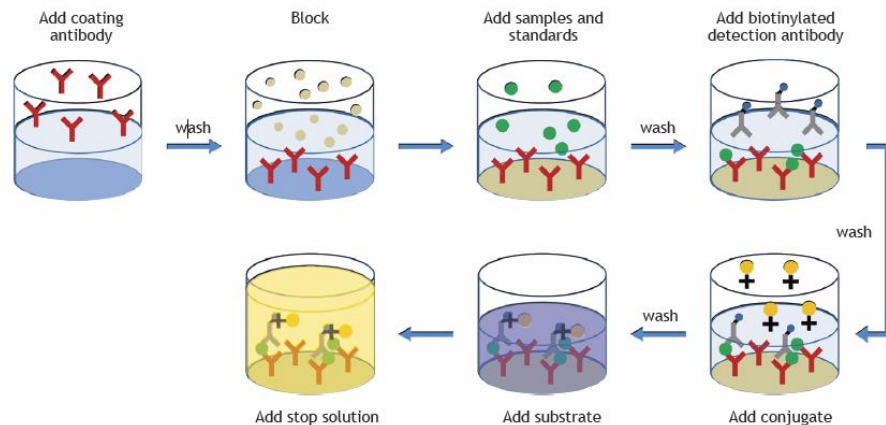
**Expiration:**

Assay Range:	2-128 pg/ml
Sensitivity:	2 pg/ml
Specificity:	Native and Recombinant Human TNF-a*
Calibration:	Recombinant Human TNF-a
Type of sample:	Plasma, serum, cell culture supernatant

- Addition of a 1000-fold molar excess of soluble TNF-a receptors (RI/RII) has 10% interference on the ELISA results.

### Principle of the Test

Cell Science's Human TNF-a ELISA assay is a simple and sensitive sandwich immunoassay for the determination of human TNF-a in biological fluids such as cell culture supernatant, plasma, or serum. The assay utilizes a coating antibody specific for TNF-a coated on the wells of a 96-well microtiter plate. The wells are washed and blocked. Standards and samples are added to the wells and any analyte present binds to the immobilized coating antibody. After washing off excess/unbound materials, the bound analyte is allowed to associate with a biotinylated detection antibody specific for TNF-a, the wells are washed again and a streptavidin-HRP conjugate (SSP) is added to the antibody-antigen-antibody complex. After another wash, a chromogenic substrate (TMB) is introduced, which produces a blue-colored product of which the intensity is related to the amount of analyte in the sample. A sulfuric acid solution is added to stop the enzymatic reaction (changing the color to yellow) and OD is read at 450 nm.



### Warnings and Precautions

When cytokine, chemokine or granzyme levels are determined in blood components or other biological materials, please note that all these materials should be considered as potentially infectious and handled with the usual precautions under Bio-Hazard conditions. Follow universal precautions such as established by the US government agencies, Centers for Disease Control and Prevention and Occupational Safety and Health Administration, when handling and disposing of (potentially) infectious waste (see CDC/NIH manual "Biosafety in Microbiological and Biomedical Laboratories", 2009.)



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## Hazard Information:

TMB (tetramethylbenzidine) is irritating to eyes, respiratory system and skin. In case of contact with eyes, rinse immediately with plenty of water for at least 15 minutes, assuring adequate flushing by separating eyelids, and seek medical help. In case of ingestion or contact with skin, rinse mouth (if person is conscious) or wash skin with soap and water, remove contaminated clothing and shoes, and seek medical help.

## Reagents and materials supplied with the kit:

Items	Quantity (5 Plates)	Storage conditions
CKH111-A Coating antibody (lyophilized)	1 vial	4°C
CKH111-B Standard (lyophilized)	5 vials ( <b>7.9 ng/vial</b> )	4°C
CKH111-C Biotinylated detection antibody (lyophilized)	1 vial	4°C
CKH111-D SSP conjugate (lyophilized)	1 vial	-20°C to -80°C in the dark
CKH111-E BSA Stock solution (10%)	2 vials (2 x 12 mL)	4°C
CKH111-F Cytokine stabilization buffer (CSB)*	1 vial (5 mL)	4°C
CKH111-G Tween-20	1 vial (5 mL)	Room Temperature (RT) in the dark
CKH111-H TMB substrate solution	2 vials (2 x 30 mL)	4°C in the dark
CKH111-I Stop solution (0.175 M H <sub>2</sub> SO <sub>4</sub> )	2 vials (2 x 30 mL)	4°C
CKH111-P ELISA plates (not pre-coated)	8	Room Temperature (RT)
CKH111-S Adhesive cover slips	10	Room Temperature (RT)

\* For use with serum and plasma samples only, see section "Preparation of Samples".

## Storage and Stability:

Coating antibody and biotinylated detection antibody - The vials with lyophilized coating and biotinylated detection antibody can be safely stored at 4°C until kit expiration date. After reconstitution, the antibodies are stable for at least 12 months at 4°C when kept sterile. However, it is recommended to divide the reconstituted antibody solutions into small aliquots for single use. These aliquots should be stored at -20°C to -80°C (stable for at least 2 years).

Standard - The vials with lyophilized standards can be safely stored at 4°C until kit expiration date. These vials are for single use only.

SPP conjugate - The vial with lyophilized SPP conjugate is stable until kit expiration date when stored at -20°C in the dark. After reconstitution, the reagent is stable for at least 2 months at 4°C when kept sterile and protected from light. However, it is strongly recommended to divide the solution into small aliquots for single use. These aliquots should be stored at -20°C to -80°C in the dark (stable for at least one year).

TMB Substrate - The ready-to-use TMB substrate solution should be stored at 4°C and is stable until kit expiration date. Avoid exposure to light, heat and contamination with metal ions or peroxidase.

### BSA stock solution and cytokine stabilization buffer

The vials with BSA stock solution and Cytokine stabilization buffer can be safely stored at 4°C until kit expiration date. After opening, these solutions are stable for at least 6 months when kept sterile.

Tween-20 - Tween-20 should be stored at RT in the dark and is stable until kit expiration date.

Stop Solution - The ready-to-use Stop solution can safely be stored at 4°C and is stable until kit expiration date.



## Materials/reagents required but not provided:

- PBS, pH 7.4 (prepare as described in "Preparation Solutions and Reagents" section or use commercially available PBS (pH 7.4).
- Sterile distilled water and ice
- Pipetting devices for the accurate delivery of volume required for the assay performance.
- Tubes and containers/plates to make solutions.
- Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
- Reading device for microtiter-plate (wavelength set to 450 or 650 nm).

## Specimen Collection and Handling:

Specimens should be clear, non-hemolyzed and non-lipemic. Excessive hemolysis and the presence of large clots or microbial growth in the sample may interfere with the performance of the test.

- **Cell culture supernatant:** remove any particulate matter by centrifugation.
- **Serum:** use a clot tube and allow sample to clot for 30-45 min at RT, then centrifuge for 10-15 minutes at 1,000 to 2,000 x g at RT and collect serum immediately.
- **Plasma:** collect plasma by using anticoagulant, such as EDTA or heparin. Mix well immediately after collection. Centrifuge for 10-15 min at 1,000-2,000 x g at RT and collect plasma.

Samples should be aliquoted and stored frozen at -20 °C to prevent cytokine degradation. If samples are run within 24 hours, they may be stored at 2-8 °C. Avoid repeated freeze-thaw cycles. Do not heat serum or plasma samples. Prior to assay, frozen samples should be completely thawed and mixed well.

## Preparation of Solutions and Reagents:

### PBS

- PB stock: dissolve 96.0 g of Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O plus 17.5 g of KH<sub>2</sub>PO<sub>4</sub> in 1 L distilled water, adjust pH to 7.4 and filter solution (0.2 µm). Store solution at RT. The PB stock is 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.

### Wash buffer

- PBS containing 0.05% Tween-20 (add 0.5 mL of Tween-20 to 1 L PBS). The volume is dependent on the washing procedure (manual or automatic washing).

### Blocking buffer

- PBS containing 1% BSA. For one ELISA plate, 2 mL BSA stock solution (10%) is gently but thoroughly mixed 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 with at least 250 ml PBS under sterile conditions. Add 12.5 mL of BSA stock solution (10%) and 125 µL of Tween-20 (1:2,000) 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, you will need 20 mL of Dilution buffer 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

- Reconstitute the lyophilized antibody by injecting 250  $\mu$ L of sterile distilled water into the vial. Mix the solution gently for approximately 15 seconds and allow it to stand for 5 minutes at RT. Avoid vigorous shaking.
- For one ELISA plate: 50  $\mu$ L is gently but thoroughly mixed with 5 mL PBS (1:100).
- **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).**

## Standard

- **Each vial contains 7.9 ng recombinant TNF- $\alpha$  standard.** For maximum recovery, the vial with lyophilized standard should be reconstituted in 0.5 mL sterile distilled water, gently mixed for approximately 15 seconds, and allowed to stand for 5 minutes at RT. Avoid vigorous shaking. The reconstituted standard (stock solution) is placed on crushed ice and is immediately diluted as indicated below (preferentially within one hour).
- **Note: The quantity (expressed in ng/vial) of the Standard is indicated on the vial and is variable for each kit and lot. After reconstitution, the concentration can be calculated as follows: divide the quantity (indicated on the vial) by the volume used for reconstitution. For example, the concentration of a standard containing 5 ng/vial will be 10 ng/mL (= 10,000 pg/mL) after reconstitution in 0.5 mL distilled water.**

## Biotinylated detection antibody

- Reconstitute the lyophilized antibody by injecting 500  $\mu$ L of sterile distilled water into the vial. Mix the solution gently for approximately 15 seconds and allow it to stand for 5 minutes at RT. Avoid vigorous shaking.
- For one ELISA plate: 100  $\mu$ L is gently and thoroughly mixed with 10 mL Dilution buffer (1:100 dilution).

## SPP conjugate

- Reconstitute the contents of the vial by injecting 0.5 mL of sterile distilled water into the vial. Mix the solution gently for approximately 15 seconds and allow it to stand for 5 minutes at 4  $^{\circ}$ C in the dark. Avoid vigorous shaking.
- For one ELISA plate: 100  $\mu$ L is gently and thoroughly mixed with 10 mL Dilution buffer (1:100).

## TMB substrate solution (ready-to-use)

- Bring TMB substrate solution to RT prior to use.

## Stop solution (ready-to-use)

- Bring Stop solution to RT prior to use.

## Sample Preparation

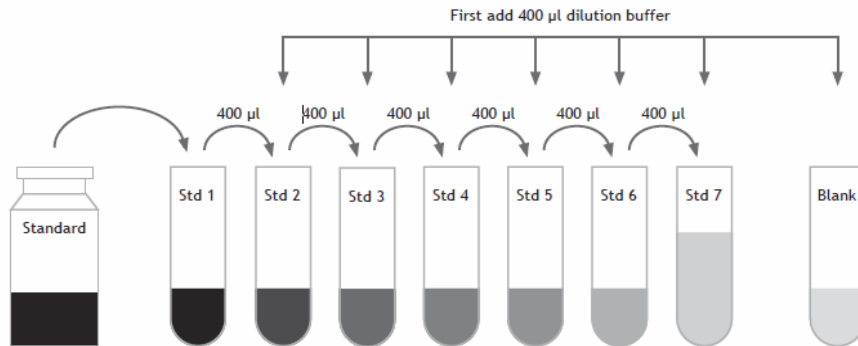
- Dilute samples in Dilution buffer (at least 1:1). We recommend analyzing a series of dilutions of the sample to ensure that sample measurements fall within the standard curve range (see also section "Interpretation of Results").
- When measuring cytokines in serum or plasma, add 1/20 volume of CSB (ready-to-use) to 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.
- It is recommended to analyze samples in triplicate.

## Preparing the Standards



With a standard curve, the analyte concentrations in the unknown samples can be determined. The standard curve is generated from the data of 7 two-fold serial dilution (Std 1-7) of the Standard. The recommended assay range for each specific ELISA kit can be seen in the Specifications Table at the top of the first page. **It is recommended to test the standard dilutions (Std 1-7) in triplicate.**

- Take 8 tubes and add 400  $\mu$ L Dilution buffer to 7 of these tubes (Std 2 to Std 7 and Blank).
- Prepare in the remaining tube (Std 1) the highest concentration to be used in the standard curve (see Specifications Table) by mixing an appropriate volume of Standard with Dilution buffer. The final volume of Std 1 should be 800  $\mu$ L. Allow the mixture to stand for at least 15 seconds before using in further dilutions.
- Perform two-fold serial dilutions: transfer 400  $\mu$ L diluted standard from Std 1 to the next tube (Std 2), mix well and repeat this step until Std 7 (see figure below).



## Notes:

- If less than 10  $\mu$ L of the Standard is needed to make Std 1, it is recommended to dilute the Standard 10 times in Dilution buffer (mix 10  $\mu$ L Standard with 90  $\mu$ L Dilution buffer) and use this to make Std 1.
- A standard curve, including blank, should be run on each ELISA plate.
- Use vials with Standard only once.
- **It is recommended to test standard dilutions in triplicate.**
- Standard dilutions should be used as soon as possible (preferentially within one hour).
- Depending on the biological origin of the unknown samples, other appropriate dilution buffers may be used for the preparation of the standard curve, depleted with the endogenous protein to be quantified (e.g. cell culture medium, serum.)

## Directions for Washing

- Incomplete washing of the wells will adversely affect the assay. All washing steps should be performed with Wash buffer.
- Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering the tip of an aspiration device into each well (without touching the bottom). **After aspiration, fill the wells with at least 250  $\mu$ L Wash buffer and then aspirate the liquid. Repeat these steps at least six times.** After washing, the plate is inverted and tapped dry on absorbent tissue paper. Alternatively, the Wash buffer may be put into a squirt bottle. If a squirt bottle is used, empty the wells and flood the plate with Wash buffer, completely filling all wells. Repeat these steps at least six times. After washing, the plate is inverted and tapped dry on absorbent tissue paper.
- When using an automated washing device, follow operating instructions carefully.



## Assay procedure

**Note:** All solutions should reach RT prior to use.

1. Add 50  $\mu$ L of diluted coating antibody solution to each well of the ELISA plate and fill up to 100  $\mu$ L with PBS. Seal the plate to prevent evaporation.
2. Incubate overnight at 4  $^{\circ}$ C
3. Remove the coating antibody solution and wash the wells at least 6 times with Wash buffer (see Wash Directions)
4. Add 200  $\mu$ L of Blocking buffer to each well.
5. Seal the plate and incubate for 1 hour at RT.
6. Prepare blank, samples, standard dilutions.
7. Remove the Blocking buffer (do not wash the wells).
8. Add 100  $\mu$ L of diluted standards, blank, or samples to each well.
9. Seal the plate and incubate for 2 hours at RT (or alternatively overnight at 4  $^{\circ}$ C).
10. Remove standards/samples and wash the wells at least 6 times with Wash buffer.
11. Add 100  $\mu$ L of diluted detector antibody solution to each well.
12. Seal the plate and incubate for 1 hour at RT.
13. Remove detection antibody solution and wash the wells at least 6 times with Wash buffer.
14. Add 100  $\mu$ L of diluted SPP conjugate to each well.
15. Seal the plate and incubate for 1 hour at RT (protected from light.)
16. Remove SPP conjugate and wash the wells at least 6 times with Wash buffer.
17. Add 100  $\mu$ L of TMB substrate solution into each well.
18. Leave the plate protected from light for 20 minutes at RT. The substrate produces a soluble end product that is blue in color that absorbs at 650 nm.
19. After the substrate incubation, do not empty the wells. Stop the reaction by adding 100  $\mu$ L of Stop solution (resulting in a yellow solution) and read the plate at 450 nm within 30 minutes.

## Data analysis

In each experiment, a standard curve needs to be run, consisting of 7 standard dilutions, from which a concentration-response relationship is generated. Construct the standard curve by plotting the mean OD of each standard dilution (Std 1-7) minus the mean Blank (see formula below) on the y-axis against the corresponding concentration on the x-axis.

**Formula:** Calculated adjusted Standard OD = mean OD<sub>Std 1-7 samples</sub> – mean OD<sub>Blank</sub>

Most laboratories have (plate reader) software that allows various methods of curve fitting. Since ELISA data are essentially sigmoidal rather than linear, we recommend using the 4 or 5-parameter logistic fit for quantitative analysis of the samples. Alternatively, a linear regression curve may be acceptable for the linear portion of the curve consisting of at least 3 concentrations.

After selection of the regression model, the analyte concentrations in unknown samples can be interpolated from the standard curve. The OD value of the sample should fall within the standard curve. Samples showing an OD below the lowest concentration of the standard curve need to be re-analyzed at a lower dilution. Samples showing an OD that exceeds the highest concentration of the standard curve need to be re-analyzed at a higher dilution.

If samples have been diluted, the calculated concentration must be multiplied by the dilution factor.

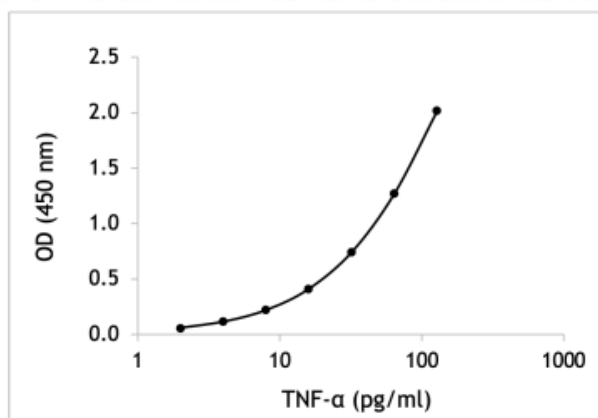
## Typical Data



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Representative standard curve of Human TNF-a

## Troubleshooting

Problem	Possible cause	Solution
Poor consistency of replicates	Inaccurate pipetting	- Ensure accurate pipetting of volume and avoid air bubbles. - Check pipettes.
	Inadequate mixing of reagents	- Mix reagents adequately.
	Inadequate washing	- Increase the stringency of washes (particularly after the 'detection antibody' incubation step). - Check function of the plate washer.
	Evaporation of solutions	- Ensure precise sealing of the plate.
	Non-homogenous samples or with high particulate matter	- Mix samples thoroughly and remove particulates by centrifugation.
OD <sub>blank</sub> values higher than 0.3	Incubation time of TMB substrate solution is too long	- Shorter incubation time of TMB substrate.
	Incubation temperature of TMB substrate solution is too high	- Decrease incubation temperature of TMB substrate.
	Working solutions were contaminated	- Solutions should be clear and colorless. Use a clean container before addition into wells.
	Detection antibody or conjugate dilution was too concentrated or left too long on the plate	- Ensure proper dilution of detection antibody or conjugate and incubation time.
No signal or low OD values for standards	Improper storage of reconstituted SPP	- Avoid prolonged exposure to light and heat. - Avoid storage at RT.
	Incorrect incubation times or temperature	- Ensure sufficient incubation times. - Reagent solutions should be at RT before use.
	Improper quality or pH of distilled water	- Use distilled water, and not tap water. - Check quality and pH of distilled water.
	Improper antibody, SPP or standard dilution	- Ensure proper dilution of antibody, SPP and standard.
	Degradation of antibodies or SPP	- Follow recommended storage conditions.
	Overly high washing / aspiration pressure from automated plate washer.	- Check function of washing system or utilize manual washing.
	Working solutions contain sodium azide	- Avoid adding sodium azide in solutions, as this is a 'peroxidase activity' inhibitor.
Poor standard curve (linearity and dynamic range)	Improper standard dilutions	- Ensure proper dilution of standards (follow 'two-fold dilutions' guidelines).
	Inaccurate pipetting	- Ensure accurate pipetting of volume. - Check pipettes.

**NOT FOR HUMAN USE. FOR RESEARCH ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE.**

