BD OptEIA™

Rat TNF ELISA Kit

Instruction Manual

Catalog No. 560479

BD

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Introduction

Tumor necrosis factor (TNF, formerly known as TNF-α) is a potent mediator of immune and inflammatory responses.[1-2] TNF is produced by many activated cell types including monocytes, macrophages, astrocytes, granulocytes, T and B lymphocytes, NK cells, keratinocytes, fibroblasts, and certain tumor cells. TNF exerts many regulatory influences on the activation, growth, and differentiation of leukocytes and other cells. For example, TNF can costimulate the proliferation of activated T and B lymphocytes, upregulate the expressed levels of MHC class I and class II molecules by various cell types, as well as induce the expression of adhesion molecules by endothelial cells. TNF is selectively cytotoxic for some transformed cell lines and can exert cytotoxic effects against certain solid tumors. In vivo, TNF serves as a primary mediator in protective immune responses against microbial and viral pathogens. However, TNF has also been implicated as a central mediator in a number of pathologic responses including septic shock, cachexia, and autoimmune diseases.

Activated cells initially express TNF as transmembrane proteins that associate to form homotrimeric complexes. After proteolytic cleavage, the extracellular region of membrane TNF sheds as a soluble homotrimer. Both Membrane and soluble TNF homotrimers are biologically active, whereas monomeric TNF is not. The mature rat TNF monomer contains 157 amino acid residues has a predicted size of ~17 kDa.[3] TNF exerts its biological activities by binding and signaling through membrane Type I and II TNF Receptors (TNFRI and TNFRII). Interestingly, TNF binds to soluble forms of TNFR (sTNFRI and sTNFRII) which are naturally shed by activated cells and can modulate biological activities of TNF.

The BD OptEIA™ Rat TNF ELISA Kit is for the quantitative determination of TNF in rat serum, plasma, and cell culture supernatant.

Principle of the Test

The BD OptEIA test is a solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay). It utilizes a monoclonal antibody specific for rat TNF coated on a 96-well plate. Standards and samples are added to the wells, and any TNF present binds to the immobilized antibody. The wells are washed, and biotinylated anti-rat TNF antibody is added, producing an antibody-antigen-antibody "sandwich." After a second wash, streptavidin-horseradish peroxidase conjugate is added. The wells are again washed and TMB substrate solution is added, which produces a blue color in direct proportion to the amount of TNF present in the initial sample. The Stop Solution changes the color from blue to yellow, and the wells are read at 450 nm.

Reagents Provided

Antibody Coated Wells:	2 plates of 96 breakable wells (12 strips × 8 wells) coated with anti-rat TNF monoclonal antibody
Detection Antibody:	30 mL of biotinylated anti-rat TNF phage Fab antibody with fetal bovine serum (FBS) and with 0.1% ProClin®-150 as a preservative
Standards:	4 vials of lyophilized recombinant rat TNF
Enzyme Concentrate (250×):	150 µL of 250× concentrated Streptavidin-horseradish peroxidase conjugate with bovine serum albumin (BSA)* and ProClin®-150 as a preservative
Standard/Sample Diluent:	30 mL of animal serum* with 0.09% sodium azide as preservative
Enzyme Diluent:	30 mL of a buffered protein base with 0.15% ProClin®-150 as a preservative
ELISA Diluent:	12 mL of a buffered protein base with 0.09% sodium azide as preservative
Wash Concentrate (20×):	100 mL of 20× concentrated detergent solution with ProClin®-150 as a preservative
TMB One-Step Substrate Reagent:	30 mL of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution
Stop Solution:	13 mL of 1 M phosphoric acid
Plate Sealers:	4 sheets with adhesive backing

**Source of all serum proteins is from USDA inspected abattoirs located in the United States*

Materials Required but not Provided

- Microplate reader capable of measuring absorbance at 450 nm
- Precision pipettes to deliver 50 µL and 100 µL volumes
- Adjustable 1 mL, 5 mL, 10 mL, 25 mL pipettes for reagent preparation
- Deionized or distilled water
- Wash bottle or automated microplate washer
- Log-log graph paper or computer and software for ELISA data analysis
- Tubes to prepare standard dilutions
- Laboratory timer
- Absorbent paper

Storage Information

1. Store the unopened kit at 2 - 8°C. Do not use the kit after the expiration date.
2. Before use, bring all reagents to room temperature (18 - 25°C). Immediately after use, return to proper storage conditions.
3. Lyophilized standards are stable until the kit expiration date. After reconstitution, use freshly reconstituted standard within 12 hours (stored at 2 - 8°C).

Warnings and Precautions

1. Reagents that contain preservatives may be toxic if ingested, inhaled, or brought in contact with skin.
2. Avoid contact of skin, eyes, or clothing with Stop Solution or Substrate Reagents.
3. Handle all serum and plasma specimens in accordance with CLSI guidelines for preventing transmission of blood-borne infections.
4. Standard/Sample Diluent and ELISA Diluent contain less than 0.1% sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

5. ***Warning***

Wash Concentrate (20X) (component 51-9003738) contains 0.002% (w/w), Enzyme Diluent (component 51-2718KD) contains 0.003% (w/w), Rat TNF Lyophilized Standard (component 51-27266E) contains 0.03% (w/w) and Detection Antibody Biotin Anti-Rat TNF (component 51-9006282) contains 0.003% (w/w) of a CMIT/MIT mixture (3:1), which is a mixture of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC No 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC No 220-239-6] (3:1).

Hazard statements

May cause an allergic skin reaction.

Precautionary statements

Wear protective gloves / eye protection.

Wear protective clothing.

Avoid breathing mist/vapours/spray.

If skin irritation or rash occurs: Get medical advice/attention.

IF ON SKIN: Wash with plenty of water.

Dispose of contents/container in accordance with local/regional/national/international regulations.

6. ***Danger***

Stop Solution (component 51-2608KC) contains 15.23% phosphoric acid (w/w).

Hazard statements

Causes severe skin burns and eye damage.

Precautionary statements

Wear protective gloves / eye protection.

Wear protective clothing.

IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.

IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do.

Continue rinsing.

IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.

Dispose of contents/container in accordance with local/regional/national/international regulations.

Specimen Collection and Handling

Specimens should be clear, non-hemolyzed, and non-lipemic. Samples with expected values greater than 2000 pg/mL should be diluted with Standard/Sample Diluent prior to running the assay.

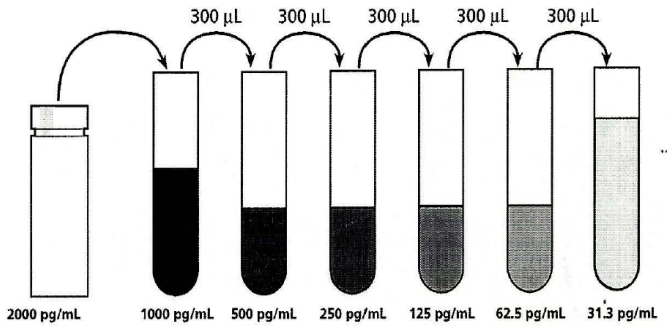
Cell culture supernatants: Remove any particulate material by centrifugation and assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Serum: Use a serum tube (eg, BD Vacutainer® Cat. No. 366430) and allow samples to clot for 30 minutes, then centrifuge for 10 minutes at 1000 × *g*. Remove serum and assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using heparin, EDTA, or citrate as anticoagulant. Centrifuge for 10 minutes at 1000 × *g* within 30 minutes of collection. Assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Reagent Preparation

1. Bring all reagents to room temperature (18 - 25°C) before use.
2. Standards
   a. Reconstitute 1 vial lyophilized Standard with the required volume (noted on vial label) of Standard/Sample Diluent to prepare a 2000 pg/mL stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions. Vortex to mix.
   b. Add 300 µL Standard/Sample Diluent to 6 tubes. Label the tubes as 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, and 31.3 pg/mL.
   c. Perform serial dilutions by adding 300 µL of each standard to the next tube and vortexing between each transfer. The undiluted standard serves as the high standard (2000 pg/mL). The Standard/Sample Diluent serves as the zero standard (0 pg/mL).



3. Wash Buffer

   Note: If the Wash Concentrate contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute the required quantity of 20× Wash Concentrate with deionized or distilled water and mix. (To prepare 2,000 mL, add 100 mL of Wash Concentrate to 1,900 mL water. At least 500 mL solution should be prepared for a full 96-well plate).

4. TMB One-Step Substrate Reagent

   No more than 15 minutes prior to use, add required volume of TMB One-Step Substrate Reagent to a clean tube or reservoir. To prevent contamination, pipette out from the tube/ reservoir instead of directly from the bottle. Avoid prolonged exposure to light or contact with metal, air, or extreme temperature as color may develop.

Assay Procedure

1. Bring all reagents and samples to room temperature (18 - 25°C) prior to use. It is recommended that all standards and samples be run in duplicate.
2. Remove the required quantity of test strips/wells and place in well holder.

   Note: Wells are provided in breakable 8-well strips. Strips may be "broken" into individual wells, replaced in a well holder, and assayed. Return any unused wells to sealed pouch for 2 - 8°C storage.

3. Pipette 50 µL of ELISA Diluent into each well.
4. Pipette 100 µL of each standard (see ***Reagent Preparation*, step 2**) and sample into appropriate wells. Gently shake/tap the plate for 5 seconds to mix. Cover wells with Plate Sealer and incubate for 2 hours at room temperature.
5. Decant or aspirate contents of wells. Wash wells by filling with at least 300 µL/well prepared Wash Buffer (see ***Reagent Preparation*, step 3**), followed by decanting/aspirating. Repeat the wash 4 times for a total of 5 washes. After the last wash, blot the plate on absorbent paper to remove any residual buffer. Complete removal of liquid is required for proper performance.
6. Add 100 µL of Detection Antibody to each well. Cover wells with Plate Sealer and incubate for 1 hour at room temperature.

7. a. Prepare Enzyme Working Reagent.

   b. Pipette the required volume of Enzyme Diluent into a clean tube or flask. Add in the required quantity of Enzyme Concentrate (250×) and vortex or mix well. For a full 96-well plate, add 48 µL of Enzyme Concentrate into 12 mL of Enzyme Diluent.

8. Wash wells as in **Step 5**.

9. Add 100 µL of Enzyme Working Reagent (see **step 7** above) to each well. Cover wells with Plate Sealer and incubate for 30 minutes at room temperature.

10. Wash wells as in **Step 5**, but a total of 7 times.

    Note: In this final wash step, soak wells in wash buffer for 30 seconds to 1 minute for each wash. Thorough washing at this step is very important.

11. Add 100 µL of TMB One-Step Substrate Reagent to each well. Incubate plate (without Plate Sealer) for 30 minutes at room temperature in the dark.

12. Add 50 µL of Stop Solution to each well.

13. Read absorbance at 450 nm within 30 minutes of stopping the reaction. If wavelength correction is available, subtract the optical density readings at 570 nm from readings at 450 nm.

    A standard curve is required in each assay run.

Assay Procedure Summary

1. Add 50 µL of ELISA Diluent to each well.
2. Add 100 µL of standard or sample to each well.
   Incubate 2 hours at room temperature.
3. Aspirate and wash 5 times.
4. Add 100 µL of Detection Antibody to each well.
   Incubate 1 hour at room temperature.
5. Aspirate and wash 5 times.
6. Add 100 µL of Enzyme Working Reagent to each well.
   Incubate 30 minutes at room temperature.
7. Aspirate and wash/soak 7 times.
8. Add 100 µL of TMB One-Step Substrate Reagent to each well.
   Incubate 30 minutes at room temperature.
9. Add 50 µL of Stop Solution to each well.
   Read at 450 nm within 30 minutes.
   λ correction 570 nm.

Calculation of Results

Calculate the mean absorbance for each set of duplicate standards, controls, and samples. Subtract the mean zero standard absorbance (ie, plate background) from each.

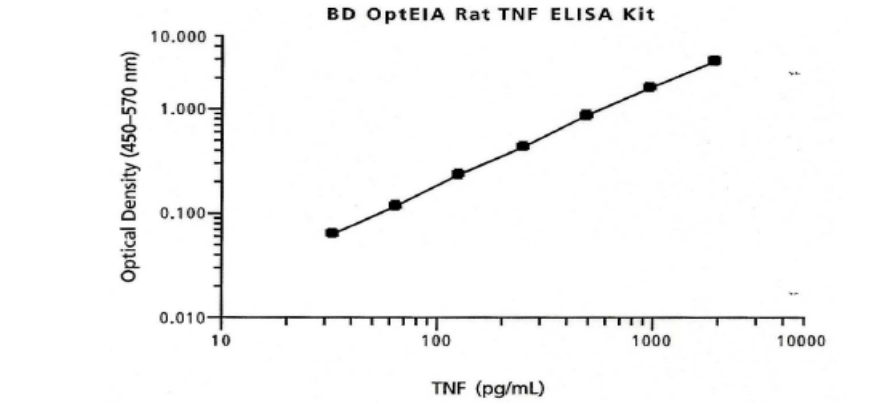
Plot the standard curve on log-log graph paper, with TNF concentration on the x-axis and absorbance on the y-axis. Draw the best fit straight line through the standard points.

To determine the TNF concentration of the unknowns, find the unknowns' mean absorbance value on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the TNF concentration. If samples were diluted, multiply the interpolated TNF concentration by the dilution factor.

Computer-based curve-fitting statistical software may also be employed.

Typical Data

This standard curve is for demonstration only. A standard curve must be run with each assay.



Concentration (pg/mL)	OD1	OD2	Mean	Zero Standard Subtracted
0	0.084	0.086	0.085	0.000
31.3	0.141	0.137	0.139	0.054
62.5	0.187	0.183	0.185	0.100
125	0.281	0.286	0.283	0.199
250	0.467	0.457	0.462	0.377
500	0.813	0.825	0.819	0.734
1000	1.420	1.491	1.455	1.371
2000	2.438	2.542	2.490	2.405

Limitations of the Procedure

1. This kit is intended for use as an integral unit. Do not mix reagents from different kit lots. Reagents from other manufacturers and other available clones should not be used in this kit.
2. Interference by drug metabolites, soluble receptors, or other binding proteins in specimens has not been thoroughly investigated. The possibility of interference cannot be excluded.

Performance Characteristics

Limit of Detection

The minimum detectable dose of TNF was determined to be 4.59 pg/mL. This is defined as two standard deviations above the mean optical density of 20 replicates of the zero standard.

Recovery

Three different amounts of TNF were spiked into rat serum and cell culture media. Results are compared with the same amounts of TNF spiked in Standard/Sample Diluent, as follows:

	Spike Concentration (pg/mL)	Average % Recovery	Range
Serum (n = 5)	1000 500 250	94 98 99	88 - 96 91 - 102 92 - 103
Cell culture media (n = 3)	1000 500 250	121 112 107	117 - 125 110 - 115 106 - 108

Linearity

Serum and cell culture media spiked with high concentrations of TNF were serially diluted with Standard/Sample-Diluent and run using the BD OptEIA Rat TNF ELISA kit. Results are as follows:

Dilution		Pooled Serum (n = 5)	Cell culture media (n = 3)
1:2	Average % of Expected Range	92 86 - 96	112 111- 114
1:4	Average % of Expected Range	94 84 - 101	116 114 - 119
1:8	Average % of Expected Range	92 81 - 98	114 109 - 119
1:16	Average % of Expected Range	93 87 - 103	108 105 - 110

Specificity

Cross-Reactivity: The following factors were tested using the BD OptEIA assay at 10 ng/mL, and no cross-reactivity (value ≥ 15 pg/mL) was identified.

Recombinant Human

TNF

Recombinant Mouse

IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IL-18, GM-CSF, IFN-γ, LT-α, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, sTNFRI, sTNFRII, RANTES

Recombinant Rat

IL-1α, IL-2, IL-4, IL-6, IL-10, IL-18, GM-CSF, IFN-γ

Precision

Intra-assay

Twenty-four replicates each of three different levels of TNF were tested in one plate. The following results were observed:

Number of Replicates	24	24	24
Mean Concentration	1067.2 pg/mL	546.3 pg/mL	272.0 pg/mL
SD	37.5	19.1	11.6
%CV	3.5	3.5	4.3

Inter-assay

Three different levels of TNF were tested in four different plates. The following results were observed:

Number of Replicates	31	32	32
Mean Concentration	959.4 pg/mL	484.8 pg/mL	241.3 pg/mL
SD	64.4	35.7	22.3
%CV	6.7	7.4	9.2

Standardization

The immunoassay was calibrated against recombinant rat TNF.

Experimental Results

Serum

Ten normal rat serum samples were tested in this assay. All samples measured less than 31.3 pg/mL (lowest standard level).

Cell Culture Supernatants

Splenocytes or peritoneal exudate cells (PECs) from apparently healthy, normal Lou rats were cultured in RPMI 1640 complete medium with 7.5% FBS at 1×10^6 cells/mL, and stimulated with PMA/Ionomycin (5 µg/mL) for 48 hours (1) or Con-A (5 µg/mL) for 96 hours (2). PECs were differentiated into RiCK-2 cells (3). Culture supernatants were collected and quantified for TNF using the BD OptEIA Rat TNF ELISA kit. The results are as follows:

Rat No.	TNF (pg/mL)	
1	1,667	Splenocytes, 48 hr PMA (5 ng/mL) + Ionomycin (500 µg) stimulation
2	1,013	Splenocyte, 96 hr Con A (5 µg/mL)
3	10,775	RiCK-2

Troubleshooting

Problem	Possible Source	Corrective Action
Poor Precision	• Inadequate washing / aspiration of wells • Inadequate mixing of reagents • Imprecise / inaccurate pipetting • Imprecise sealing of plate	• Check function of washing system • Ensure adequate mixing • Check / calibrate pipettes • Ensure complete sealing of plate
Poor Standard Curve	• Improper standard handling / dilution • Incomplete washing / aspiration of wells • Imprecise / inaccurate pipetting	• Ensure correct preparation of standards • Check function of washing system • Check / calibrate pipettes
Low Signal	• Inadequate reagent volumes added to wells • Incorrect incubation times / temperature • Overly high wash / aspiration pressure from automated plate-washer.	• Check / calibrate pipettes • Ensure sufficient incubation times / reagents warmed to room temperature • Utilize manual washing

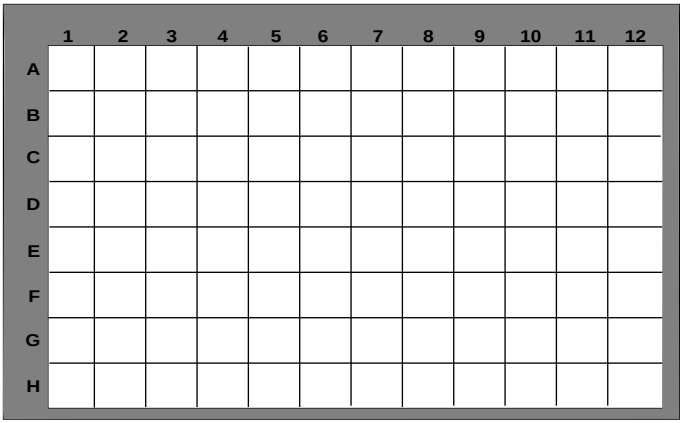
References

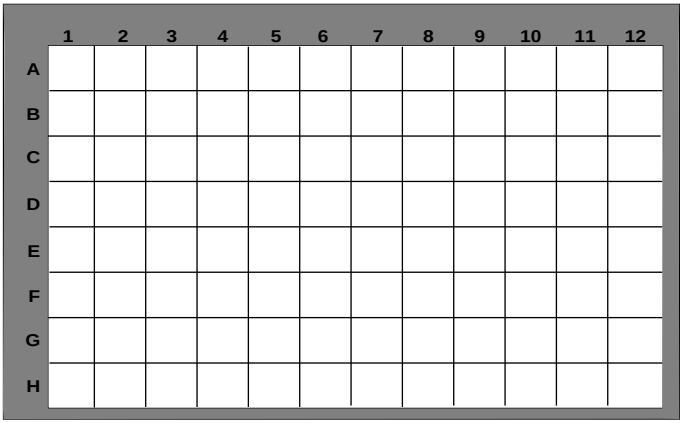
1. Beyaert R, Fiers W. Tumor necrosis factor and lymphotoxin. In: Mire-Sluis AR, Thorpe R, eds. *Cytokines*. San Diego, CA: Academic Press; 1998:335-360.

2. Ware CF, Santee S, Glass A. Tumor necrosis factor-related ligands and receptors. In: Thompson A, ed. *The Cytokine Handbook*. 3rd ed. San Diego, CA: Academic Press; 1998:549-592.

3. Kwon J, Chung IY, Benveniste EN. Cloning and sequence analysis of the rat tumor necrosis factor-encoding genes. *Gene*. 1993;132:227-236

Plate Templates





Notes

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Catalog No. 560479
Rev# 2