BD OptEIA™

Human TNF ELISA Kit II

Instruction Manual

Catalog No. 550610

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Introduction

Tumor necrosis factor (TNF, formerly known as TNF-α),[1,2] also known as cachectin, is a potent lymphoid factor which exerts cytotoxic effects on a wide range of tumor cells and certain other target cells. This cytokine is a primary regulator of inflammatory responses. TNF is produced by macrophages, neutrophils, activated T and B lymphocytes, endothelial cells, smooth muscle cells, astrocytes, natural killer cells, lymphokine-activated killer cells, and some transformed cells.[3,4]

TNF, a 17.5 kD polypeptide of 157 amino acid residues, and LT-α (lymphotoxin alpha formerly known as TNF-β) are closely related (34% a.a. homology), bind to the same cellular receptors (CD120a and CD120b), and produce similar effects. TNFs play a critical role in the body's resistance to infection by inducing fever and activating macrophages and in the destruction of certain tumors.

TNFs and Interleukin-1 function similarly. However, over-production of TNF has been associated with cytotoxic effects such as cachexia.[5]

The BD OptEIA™ ELISA Kit II format was developed for superior accuracy with serum and plasma specimens. The data that demonstrates this enhancement can be located in the Performance Characteristics "Recovery" and "Linearity" sections.

The BD OptEIA Human TNF ELISA Kit II is for the quantitative determination of human TNF in 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 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 an streptavidin-horseradish peroxidase conjugate mixed with a biotinylated anti-human TNF antibody is added, producing an antibody-antigen-antibody "sandwich". 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 microwell absorbances are read at 450 nm.

Reagents Provided

Antibody Coated Wells:	2 plates of 96 breakable wells (12 strips × 8 wells) coated with anti-human TNF monoclonal antibody
Detection Antibody:	30 mL of biotinylated anti-human TNF monoclonal antibody containing FBS* and ProClin™- 150 as preservative
Standards:	4 vials lyophilized recombinant human TNF
Enzyme Concentrate:	150 µL of 250× concentrated Streptavidin-horseradish peroxidase conjugate with BSA* and ProClin™- 150 as preservative
Standard/Sample Diluent:	30 mL of animal serum* with 0.09% sodium azide as 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
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

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Storage Information

1. Store kit at 2 - 8°C. Do not use kit after 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 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 NCCLS 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.
5. ***Warning***

   Wash Concentrate (20X) (component 51-9003738) contains 0.002% (w/w) and Detection Antibody Biotin Anti-Human TNF (component 51-27242E) 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.

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

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

   Under normal conditions of intended use, this material is not expected to be an inhalation hazard.

   Ingestion may cause severe irritation of the mouth, the esophagus and the gastrointestinal tract.

   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.

7. Enzyme Reagent (Streptavidin-horseradish peroxidase conjugate (SAv-HRP)) contains the following preservatives, 1,2-benzisothiazol-3(2H)-one (CAS: 2634-33-5) and CMIT/MIT 3:1 (CAS: 55965-84-9), 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). The concentration of this material in the enzyme reagent is not classified as hazardous per GHS and CLP. Good chemical hygiene practices are recommended when handling any laboratory chemicals.

Specimen Collection and Handling

Specimens should be clear, non-hemolyzed and non-lipemic. Samples with expected values higher than the top standard, 500 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.

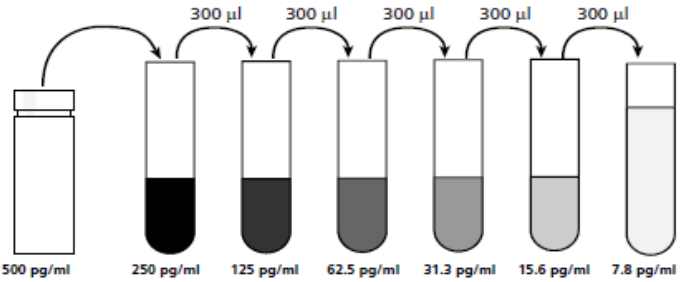
Serum: Use a serum separator tube (eg, BD Vacutainer® Cat. No. 366430) and allow samples to clot for 30 minutes, then centrifuge for 10 minutes at 1000 × *g*. Remove serum and assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using citrate, EDTA, or heparin 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 required volume (noted on vial label) of Standard/Sample Diluent to prepare a 500 pg/mL stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions. Gently vortex to mix.
   b. Add 300 µL Standard/Sample Diluent to 6 tubes. Label as 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, and 7.8 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 (500 pg/mL). The Standard/ Sample Diluent serves as the zero standard (0 pg/mL).



3. Working Detector

   Note: One-step incubation of Biotin/Streptavidin reagents. See ***Assay Procedure***, step 5.

4. Wash Buffer

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

5. 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 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. A standard curve is required in each assay run.
2. Remove required quantity of test strips/wells, place in well holder.

   Note: Wells are provided in breakable 8-well strips. Strips may be "broken" into individual wells, replaced in 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. Prepare Working Detector. Within 15 minutes prior to use, pipette required volume of Detection Antibody into a clean tube or flask. Add in required quantity of Enzyme Concentrate (250×), vortex or mix well. For a full 96-well plate, add 48 µL of Enzyme Concentrate into 12 mL of Detection Antibody.
6. Decant or aspirate contents of wells. Wash wells by filling with at least 300 µL/well prepared Wash Buffer (see ***Reagent Preparation***, step 4) followed by decanting/aspirating. Repeat wash 4 times for a total of 5 washes. After the last wash, blot plate on absorbent paper to remove any residual buffer. Complete removal of liquid is required for proper performance.
7. Add 100 µL of prepared Working Detector (see ***step 5***) to each well. Cover wells with Plate Sealer and incubate for 1 hour at room temperature.
8. Wash wells as in Step 6, 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.
9. 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.
10. Add 50 µL of Stop Solution to each well.
11. Read absorbance at 450 nm within 30 minutes of stopping reaction. If wavelength correction is available, subtract the optical density readings at 570 nm from readings at 450 nm.

Assay Procedure Summary

1. Add 50 µL ELISA Diluent to each well.
2. Add 100 µL standard or sample to each well.

   Incubate 2 hours at room temperature.
3. Aspirate and wash 5 times.

4. Add 100 µL prepared Working Detector to each well.
   Incubate 1 hour at room temperature.

5. Aspirate and wash/soak 7 times.

6. Add 100 µL TMB One-Step Substrate Reagent to each well.
   Incubate 30 minutes at room temperature.

7. Add 50 µL 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.

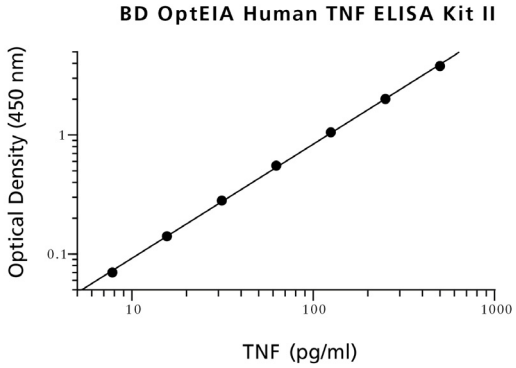
To determine the TNF concentration of the unknowns, find the unknowns' mean absorbance value on the y-axis and draw a horizontal line to the standard curve.

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	0.066	0.064	0.065	0.000
7.8	0.135	0.135	0.135	0.070
15.6	0.210	0.202	0.206	0.141
31.3	0.345	0.346	0.346	0.281
62.5	0.618	0.617	0.618	0.553
125	1.122	1.114	1.118	1.053
250	2.059	2.086	2.073	2.008
500	3.870	3.854	3.862	3.797

Limitations of the Procedure

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

Performance

Limit of Detection

The minimum detectable dose of TNF was determined to be 2.0 pg/mL. This is defined as two standard deviations above the mean optical density of 20 replicates of the zero standard.

Recovery

Three different levels of TNF were spiked into various matrices. Results are compared with same amounts of TNF spiked into Standard/Sample Diluent, as follows:

	Spike Concentration (pg/mL)	Average % Recovery	Range
Serum (n = 9)	250 125 62.5	89 86 93	81 - 95 75 - 93 78 - 109
Plasma (n = 5)	250 125 62.5	96 88 88	95 - 100 81 - 99 74 - 104
Cell culture media (n = 3)	250 125 62.5	98 106 105	88 - 106 101 - 109 100 - 110

Linearity

Various samples spiked with high concentrations of TNF were serially diluted with Standard/Sample Diluent and run in the BD OptEIA™ Human TNF ELISA Kit II. Results are as follows:

Dilution		Serum (n = 9)	Plasma (n = 5)	Cell culture media (n = 3)
1:2	Average % of Expected Range	104 95 - 116	106 100 - 111	104 101- 110
1:4	Average % of Expected Range	108 90 - 124	105 97 - 114	99 95 - 108
1:8	Average % of Expected Range	116 95 - 143	104 93 - 108	93 90 - 97
1:16	Average % of Expected Range	107 85 - 140	102 89 - 133	86 78 - 98

Specificity

Cross Reactivity: The proteins listed were spiked in Standard/Sample Diluent at 100 ng/mL to test for any cross reactivity with the BD OptEIA Human TNF ELISA Kit II assay. No cross reactivity was identified.

Interference: The factors listed below were spiked at 100 ng/mL in Standard/Sample Diluent with 100 pg/mL TNF to test for any interference with the quantitation of human TNF. No effect on assay results was observed.

Recombinant Human

IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IFN-γ, Eotaxin, G-CSF (10 ng/mL), GM-CSF, GRO, CD23, Lymphotactin (10 ng/mL), MIP-1β, MCP-1, MCP-2, MCP-3, MCP-4, NAP2, IP-10, NT-3, PDGF-AA, SCF (10 ng/mL), LT-α (TNF-β), VEGF

Recombinant Mouse

IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p70), IL-15, IFN-γ, GM-CSF, MCP-1, MIG, TCA3, TNF

Recombinant Rat

IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ, TNF, RANTES

Other

Viral IL-10 (10 ng/mL), Rabbit TNF (10 ng/mL)

Interfering Substances

The following substances at levels > 20 mg/mL were added to Standard/Sample Diluent spiked with 200 pg/mL TNF. No effect on assay results was observed.

- Bilirubin
- Human hemoglobin
- Human transferrin
- Triglycerides
- Heparin (300 units/mL)
- Sodium Citrate
- EDTA

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	213.6 pg/mL	120.2 pg/mL	66.2 pg/mL
SD	8.7	3.2	2.8
%CV	4.1	2.7	4.2

Inter-assay

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

Number of Replicates	32	32	32
Mean Concentration	223.7 pg/mL	124.2 pg/mL	63.4 pg/mL
SD	15.3	7.8	3.4
%CV	6.8	6.3	5.3

Standardization

The Human TNF immunoassay is calibrated against recombinant human TNF.

The NIBSC/WHO First International Standard 87/650 (recombinant human TNF) was evaluated in this kit. The conversion factor for NIBSC material is as follows:

NIBSC (87/650) equivalent value (IU/mL) = 0.0456 x BD OptEIA TNF value (pg/mL)

$\therefore$ 1 µg NIBSC TNF = 0.875 µg BD OptEIA TNF

Experimental Results

Serum

Twenty-eight serum samples were tested in this assay. All samples measured less than 7.8 pg/mL (lowest standard level).

Plasma

Five EDTA plasma samples were tested in this assay. Four samples measured less than 7.8 pg/mL (lowest standard level), one sample measured 21 pg/mL.

Cell Culture Supernatants

Human peripheral blood mononuclear cells or $CD8^+$ cells from seven donors were stimulated with immobilized anti-human CD3, soluble anti-human CD28, recombinant human IL-2, and recombinant human IL-4 for 2 days. Cells were harvested and recultured in complete tissue culture medium containing rhIL-2 and rhIL-4 for 3 days. Finally, the *in vitro* primed cells were harvested, washed, and restimulated with PMA and ionomycin for 4 hours, 24 hours, or 32 hours, or LPS for 45 hours (as noted). Culture supernatants were collected and quantified for TNF using a BD OptEIA Human TNF ELISA Kit II. The results are as follows:

Donor No.	TNF (pg/mL)	
1	3,422.5	PBMC, 4 hr PMA/ionomycin stimulation
2	7,589.3	PBMC, 4 hr PMA/ionomycin stimulation
3	5,786.2	PBMC, 4 hr PMA/ionomycin stimulation
4	1,622.2	PBMC, 24 hr PMA/ionomycin stimulation
5	1,802.6	PBMC, 24 hr PMA/ionomycin stimulation
5	12,053.9	CD8+ cells, 32 hr PMA/ionomycin stimulation
6	46.7	PBMC, 45 hr LPS stimulation
7	42.0	PBMC, 45 hr LPS stimulation

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

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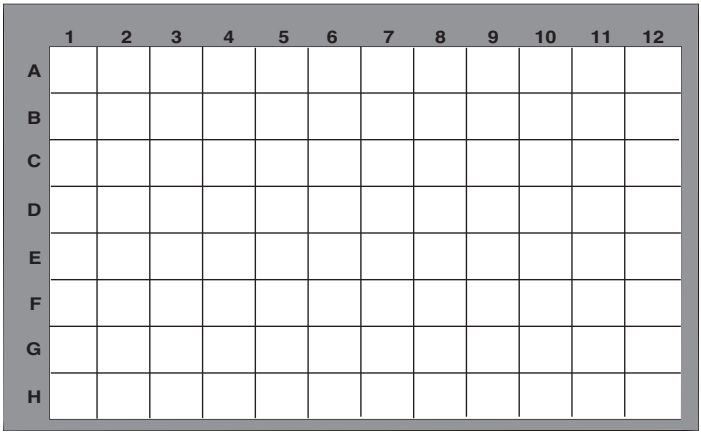
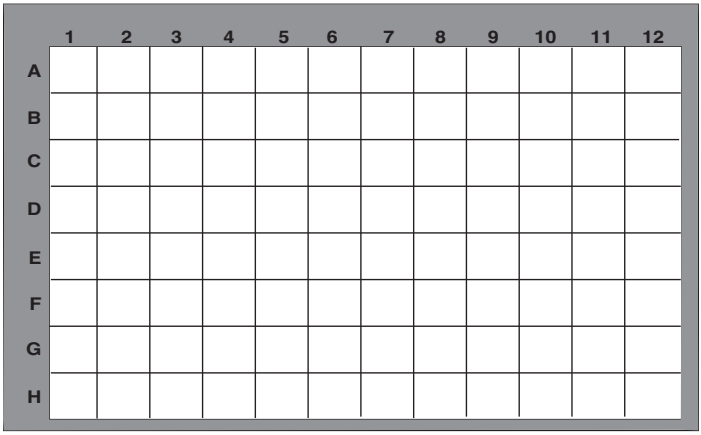
Plate Templates





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