

# Human TNF- $\alpha$ ELISA Kit

For the quantitative determination of human tumour necrosis factor alpha (TNF- $\alpha$ ) concentrations in serum, plasma, cell culture supernatant, and other biological fluids.

Catalogue Number: EL10019

*96 tests*

FOR LABORATORY RESEARCH USE ONLY  
NOT FOR DIAGNOSTIC USE



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S7.5 (05) TNF- $\alpha$

## TABLE OF CONTENTS

	Page
INTENDED USE	2
INTRODUCTION	2
PRINCIPLE OF THE ASSAY	3
REAGENTS PROVIDED	4
MATERIALS REQUIRED BUT NOT SUPPLIED	5
PRECAUTIONS	5
SAMPLE PREPARATION	6
.....Collection, Handling and Storage	6
PREPARATION OF REAGENTS	6
ASSAY PROCEDURE	8
CALCULATION OF RESULTS	9
TYPICAL DATA	9
.....Example one (Calibrator Diluent I)	10
PERFORMANCE CHARACTERICS	11
.....Intra-assay precision	11
.....Inter-assay precision	11
.....Recovery	11
.....Sensitivity	11
.....Specificity	11
.....Calibration	12
.....Testing of cell culture supernatants	12
REFERENCES & CITATIONS	12

## INTENDED USE

This Human TNF-alpha ( $\alpha$ ) ELISA Kit is to be used for the *in vitro* quantitative determination of human tumour necrosis factor alpha (TNF- $\alpha$ ) concentrations in serum, plasma, cell culture supernatant, and other biological fluids. This kit is intended FOR LABORATORY RESEARCH USE ONLY and not for use in diagnostic or therapeutic procedures.

## INTRODUCTION

Tumour Necrosis Factor alpha (TNF- $\alpha$ ), also known as cachectin was initially named for its remarkable ability to cause hemorrhagic necrosis of tumours in mice.<sup>1</sup> The TNF- $\alpha$  gene located on chromosome 6 encodes a 233 amino acid (a.a.) prohormone bound to the plasma membrane, with the mature form (157 a.a., 17 kDa) exposed on the extracellular surface. Soluble, mature TNF- $\alpha$  is released upon cleavage of the C-terminal.<sup>2,3</sup> The primary source *in vivo* of TNF- $\alpha$  is thought to be the monocyte/macrophage but various cell types are known to express this cytokine such as lymphocytes, basophils, eosinophils, mast cells, NK cells, T cells, B cells, keratinocytes, Kupffer cells, astrocytes, and some types of tumours. TNF- $\alpha$  is produced upon stimulation with cytokines such as IL-1, IL-2, GM-CSF, TNF- $\alpha$  itself and with bacterial lipopolysaccharide (LPS) which is a potent inducer.<sup>4</sup> TNF- $\alpha$  once produced and secreted will bind to TNF- $\alpha$  receptors (TNF- $\alpha$  R1, 55 kDa and TNF- $\alpha$  R2, 75 kDa), located on the plasma membrane of most cells throughout the body except the red blood cell.<sup>5</sup> The two TNF- $\alpha$  receptors deliberate the biological effects of TNF- $\alpha$ . It has been reported that TNF- $\alpha$  R1 is responsible for mediating LPS toxicity<sup>6</sup> and cell cytotoxicity<sup>7</sup> and TNF- $\alpha$  R2 is involved in cellular proliferation.<sup>8</sup> Various pathological conditions are associated with the production of high levels of TNF- $\alpha$ . These include septic shock syndrome, cachexia (e.g. HIV, tuberculosis, cancer), autoimmune diseases, hepatitis, leukemia, myocardial ischaemia, organ transplantation rejection, multiple sclerosis, rheumatoid arthritis, and meningococcal septicemia.<sup>4,9,10,11</sup> Many people die annually from a complication of infectious disease called septic shock syndrome which is triggered by TNF- $\alpha$ . In many cases elevated TNF- $\alpha$  serum levels predict a higher mortality.<sup>12</sup> TNF- $\alpha$  is a pleiotropic cytokine that can induce disease through TNF- $\alpha$  toxicity (tissue injury, catabolic illness, and mediating shock) and improve host defense mechanisms (stimulating inflammation and increasing immune cell function).<sup>4</sup> In the future, therapies may be developed by blocking TNF- $\alpha$  harmful effects and enhancing TNF- $\alpha$  beneficial effects.

*This TNF- $\alpha$  ELISA is a 2.5-3 hour solid phase immunoassay readily applicable to measure TNF- $\alpha$  levels in serum, plasma, cell culture supernatant, and other biological fluids in the range of 0 to 1000 pg/mL. It showed no cross reactivity with other cytokines tested. This TNF- $\alpha$  ELISA is expected to be effectively used for further investigations into the relationship between TNF- $\alpha$  and the various conditions mentioned.*

## PRINCIPLE OF THE ASSAY

This TNF- $\alpha$  enzyme linked immunosorbent assay (ELISA) applies a technique called a quantitative sandwich immunoassay. The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to TNF- $\alpha$ . Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated monoclonal antibody preparation specific for TNF- $\alpha$  and incubated. TNF- $\alpha$ , if present, will bind and become immobilized by the antibody pre-coated on the wells and then be “sandwiched” by biotin conjugate. The microtiter plate wells are thoroughly washed to remove unbound TNF- $\alpha$  and other components of the sample. In order to quantify the amount of TNF- $\alpha$  present in the sample, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Avidin is a tetramer containing four identical subunits that each has a high affinity-binding site for biotin. The wells are thoroughly washed to remove all unbound HRP-conjugated Avidin and a TMB (3,3',5,5' tetramethyl-benzidine) substrate solution is added to each well. The enzyme (HRP) and substrate are allowed to react over a short incubation period. Only those wells that contain TNF- $\alpha$ , biotin-conjugated antibody, and enzyme-conjugated Avidin will exhibit a change in colour. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450 nm  $\pm$  2 nm.

In order to measure the concentration of TNF- $\alpha$  in the samples, this kit includes two calibration diluents (Calibrator Diluent I for serum/plasma testing and Calibrator Diluent II for cell culture supernatant testing.) According to the testing system, the provided standard is diluted (2-fold) with the appropriate Calibrator Diluent and assayed at the same time as the samples. This allows the operator to produce a standard curve of Optical Density (O.D) versus TNF- $\alpha$  concentration (pg/mL). The concentration of TNF- $\alpha$  in the samples is then determined by comparing the O.D. of the samples to the standard curve.

## REAGENTS PROVIDED

All reagents provided are stored at 2-8°C. Refer to the expiration date on the label.

		<b>96 tests</b>
1.	<b>TNF-<math>\alpha</math> MICROTITER PLATE</b> (Part EL19-1) _____ Pre-coated with anti-human TNF- $\alpha$ monoclonal antibody.	<b>96 wells</b>
2.	<b>BIOTIN CONJUGATE</b> (Part EL19-2) _____ Anti-human TNF- $\alpha$ monoclonal antibody conjugated to Biotin.	<b>6 mL</b>
3.	<b>AVIDIN CONJUGATE</b> (Part EL19-3) _____ Avidin conjugated to horseradish peroxidase.	<b>12 mL</b>
4.	<b>TNF-<math>\alpha</math> STANDARD</b> (Part EL19-4) _____ Recombinant human TNF- $\alpha$ (2 ng/vial) in a buffered protein base with preservative, lyophilized.	<b>2 vials</b>
5.	<b>CALIBRATOR DILUENT I</b> (Part EL19-5) _____ Animal serum with buffer and preservative. <i>For serum/plasma testing.</i>	<b>25 mL</b>
6.	<b>CALIBRATOR DILUENT II</b> (Part EL19-6) _____ Cell culture medium with calf serum and preservative. <i>For cell culture supernatant testing.</i>	<b>25 mL</b>
7.	<b>WASH BUFFER (20X)</b> (Part 30005) _____ 20-fold concentrated solution of buffered surfactant.	<b>60 mL</b>
8.	<b>SUBSTRATE A</b> (Part EL19-7) _____ Buffered solution with H <sub>2</sub> O <sub>2</sub>	<b>10 mL</b>
9.	<b>SUBSTRATE B</b> (Part 30007) _____ Buffered solution with TMB.	<b>10 mL</b>
10.	<b>STOP SOLUTION</b> (Part 30008) _____ 2N Sulphuric Acid (H <sub>2</sub> SO <sub>4</sub> ). Caution: Caustic Material!	<b>14 mL</b>

## MATERIALS REQUIRED BUT NOT SUPPLIED

1. Single or multi-channel precision pipettes with disposable tips: 10-100 $\mu$ L and 50-200 $\mu$ L for running the assay.
2. Pipettes: 1 mL, 5 mL, 10 mL, and 25 mL for reagent preparation.
3. Multi-channel pipette reservoir or equivalent reagent container.
4. Test tubes and racks.
5. Polypropylene tubes or containers (25 mL).
6. Erlenmeyer flasks: 100 mL, 400 mL, 1 L and 2 L.
7. Microtiter plate reader (450 nm  $\pm$  2nm).
8. Automatic microtiter plate washer or squirt bottle.
9. Sodium hypochlorite solution, 5.25% (household liquid bleach).
10. Deionized or distilled water.
11. Plastic plate cover.
12. Disposable gloves.
13. Absorbent paper.

## PRECAUTIONS

1. Do not substitute reagents from one kit lot to another. Standard, conjugate and microtiter plates are matched for optimal performance. Use only the reagents supplied by manufacturer.
2. Allow kit reagents and materials to reach room temperature (20-25°C) before use. Do not use water baths to thaw samples or reagents.
3. Do not use kit components beyond their expiration date.
4. Use only deionized or distilled water to dilute reagents.
5. Do not remove microtiter plate from the storage bag until needed. Unused strips should be stored at 2-8°C in their pouch with the desiccant provided.
6. Use fresh disposable pipette tips for each transfer to avoid contamination.
7. Do not mix acid and sodium hypochlorite solutions.
8. Human serum and plasma should be handled as potentially hazardous and capable of transmitting disease. Disposable gloves must be worn during the assay procedure, since no known test method can offer complete assurance that products derived from human blood will not transmit infectious agents. Therefore, all blood derivatives should be considered potentially infectious and good laboratory practices should be followed.
9. All samples should be disposed of in a manner that will inactivate human viruses.  
Solid Waste: Autoclave 60 min. at 121°C.  
Liquid Waste: Add sodium hypochlorite to a final concentration of 1.0%. The waste should be allowed to stand for a minimum of 30 minutes to inactivate the viruses before disposal.
10. Substrate Solution is easily contaminated. If bluish prior to use, *do not use*.
11. Substrate B contains 20% acetone. Keep this reagent away from sources of heat or flame.
12. If Wash Buffer (20X) is stored at a lower temperature (2-5°C), crystals may form which must be dissolved by warming to 37°C prior to use.

## SAMPLE PREPARATION

### COLLECTION, HANDLING AND STORAGE

- a) **Cell Culture Supernatant:** Centrifuge to remove any visible particulate material.
- b) **Serum:** Blood should be drawn using standard venipuncture techniques and serum separated from the blood cells as soon as possible. Samples should be allowed to clot for one hour at room temperature, centrifuged for 10 minutes (4°C) and serum extracted.
- c) **Plasma:** Blood should be drawn using standard venipuncture techniques and plasma collected using sodium citrate, EDTA, or heparin as an anticoagulant. To ensure optimal recovery and minimal platelet contamination, after collection there must be quick separation of plasma with less than 30 minutes on ice. Centrifuge for 10 minutes (4°C) to remove any particulate.
  - Avoid grossly hemolytic, lipidic or turbid samples.
  - Serum, plasma, and cell culture supernatant samples to be used within 24-48 hours may be stored at 2-8°C, otherwise samples must stored at -20°C to avoid loss of bioactivity and contamination. Avoid freeze-thaw cycles.
  - When performing the assay slowly bring samples to room temperature.
  - It is recommended that all samples be assayed in duplicate.
  - DO NOT USE HEAT-TREATED SPECIMENS.

### PREPARATION OF REAGENTS

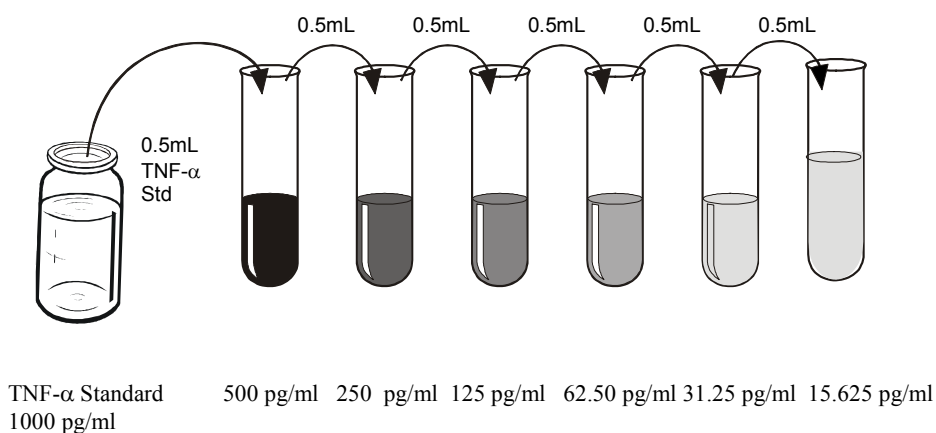
Remove all kit reagents from refrigerator and allow them to reach room temperature (20-25°C). Prepare the following reagents as indicated below.

1. **Wash Buffer (1X):** Add 60 mL of Wash Buffer (20X) and dilute to a final volume of 1200 mL with distilled or deionized water. Mix thoroughly. If a smaller volume of Wash Buffer (1X) is desired, add 1 volume of Wash Buffer (20X) to 19 volumes of distilled or deionized water. Wash Buffer (1X) is stable for 1 month at 2-8°C. Mix well before use.
2. **Substrate Solution:** Substrate A and Substrate B should be mixed together in equal volumes up to 15 minutes before use. Refer to the table provided for correct amounts of Substrate Solution to prepare.

Strips Used	Substrate A (mL)	Substrate B (mL)	Substrate Solution (mL)
2 strips (16 wells)	2.0	2.0	4.0
4 strips (32 wells)	3.0	3.0	6.0
6 strips (48 wells)	4.0	4.0	8.0
8 strips (64 wells)	5.0	5.0	10.0
10 strips (80 wells)	6.0	6.0	12.0
12 strips (96 wells)	7.0	7.0	14.0

### 3. **TNF- $\alpha$ Standard:**

- a) Two vials of Standard are provided in this kit to allow both serum/plasma and cell culture supernatant testing. Reconstitute TNF- $\alpha$  Standard with 2.0 mL of the appropriate calibrator diluent. This reconstitution produces a stock solution of 1000 pg/mL. Allow solution to sit for at least 15 minutes with gentle agitation prior to making dilutions. Use within one hour of reconstitution. The TNF- $\alpha$  standard stock solution can be stored frozen (-20°C) for up to 30 days. Avoid freeze-thaw cycles; aliquot if repeated use is expected.
- b) Use the above stock solution to produce a serial 2-fold dilution series, as described below, within the range of this assay (15.625 to 1000 pg/mL) as illustrated. Add 0.5 mL of the appropriate Calibrator Diluent to each test tube. Between each test tube transfer be sure to mix contents thoroughly. The undiluted TNF- $\alpha$  stock solution will serve as the high standard (1000 pg/mL) and the Calibrator Diluent will serve as the zero standard (0 pg/mL).





## ASSAY PROCEDURE

1. Prepare Wash Buffer and TNF- $\alpha$  Standards before starting assay procedure (see Preparation of Reagents). *It is recommended that the table and diagram provided be used as a reference for adding Standards and Samples to the Microtiter Plate.*

Wells	Contents				Wells	Contents						
<b>1A, 1B</b>	Standard 1	<b>0 pg/mL</b>	(S1)		<b>2A, 2B</b>	Standard 4	<b>125 pg/mL</b>	(S5)				
<b>1C, 1D</b>	Standard 2	<b>15.6 pg/mL</b>	(S2)		<b>2C, 2D</b>	Standard 5	<b>250 pg/mL</b>	(S6)				
<b>1E, 1F</b>	Standard 3	<b>31.2 pg/mL</b>	(S3)		<b>2E, 2F</b>	Standard 6	<b>500 pg/mL</b>	(S7)				
<b>1G, 1H</b>	Standard 4	<b>62.5 pg/mL</b>	(S4)		<b>2G, 2H</b>	Standard 7	<b>1000 pg/mL</b>	(S8)				
<b>3A, 3B</b>	<b>TNF-a samples</b>											
	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>
<b>A</b>	S1	S5	1	5	9	13	17	21	25	29	33	37
<b>B</b>	S1	S5	1	5	9	13	17	21	25	29	33	37
<b>C</b>	S2	S6	2	6	10	14	18	22	26	30	34	38
<b>D</b>	S2	S6	2	6	10	14	18	22	26	30	34	38
<b>E</b>	S3	S7	3	7	11	15	19	23	27	31	35	39
<b>F</b>	S3	S7	3	7	11	15	19	23	27	31	35	39
<b>G</b>	S4	S8	4	8	12	16	20	24	28	32	36	40
<b>H</b>	S4	S8	4	8	12	16	20	24	28	32	36	40

2. Add 100 $\mu$ L of Standard or Sample to the appropriate well of the antibody pre-coated Microtiter Plate and incubate 1 hour at room temperature.
3. Without discarding the standards and samples, add 50 $\mu$ L TNF- $\alpha$  Biotin conjugate to each wells. Mix well. Cover and incubate for 1 hour at room temperature.
4. Wash the Microtiter Plate using one of the specified methods indicated below:

Manual Washing: Remove incubation mixture by aspirating contents of the plate into a sink or proper waste container. Using a squirt bottle, fill each well completely with Wash Buffer (1X) then aspirate contents of the plate into a sink or proper waste container. Repeat this procedure four more times for a **total of FIVE washes**. After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. *Note:* Hold the sides of the plate frame firmly when washing the plate to assure that all strips remain securely in frame.

Automated Washing: Aspirate all wells, then wash plates **FIVE times** using Wash Buffer (1X). Always adjust your washer to aspirate as much liquid as possible and set fill volume at 350  $\mu$ L/well/wash (range: 350-400  $\mu$ L). After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. *It is recommended that the washer be set for a soaking time of 10 seconds or shaking time of 5 seconds between washes.*

5. Dispense 100 $\mu$ l of Avidin Conjugate to each well Mix well. Cover and incubate for 1 hour at room temperature.
6. Prepare Substrate Solution no more than 15 minutes before end of second incubation (see Preparation of Reagents).
7. Repeat wash procedure as described in Step 4.
8. Add 100 $\mu$ L Substrate Solution to each well. Cover and incubate for 15 minutes at room temperature.
9. Add 100 $\mu$ L Stop Solution to each well. Mix well.
10. Read the Optical Density (O.D.) at 450 nm using a microtiter plate reader set within 30 minutes.

#### **CALCULATION OF RESULTS**

The standard curve is used to determine the amount of TNF- $\alpha$  in an unknown sample. The standard curve is generated by plotting the average O.D. (450 nm) obtained for each of the standard concentrations on the vertical (Y) axis versus the corresponding TNF- $\alpha$  concentration (pg/mL) on the horizontal (X) axis.

1. First, calculate the mean O.D value for each standard and sample. All O.D. values are subtracted by the value of the zero-standard (0 pg/mL) before result interpretation. Construct the standard curve using graph paper or statistical software.
2. To determine the amount of TNF- $\alpha$  in each sample, first locate the O.D. value on the Y-axis and extend a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the corresponding TNF- $\alpha$  concentration. If samples generate values higher than the highest standard, dilute the samples and repeat the assay, the concentration read from the standard curve must be multiplied by the dilution factor.

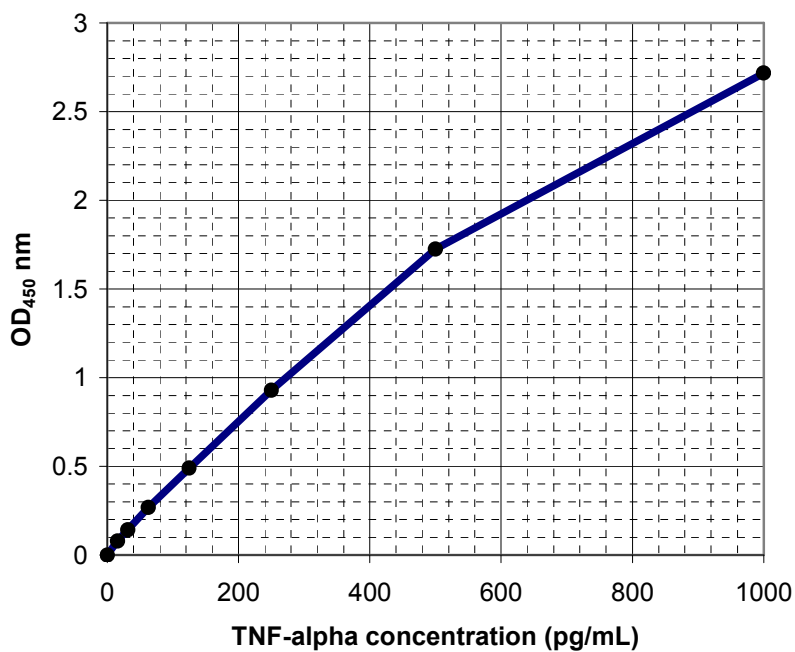
#### **TYPICAL DATA**

Results of a typical standard run of a TNF- $\alpha$  ELISA are shown below. Any variation in standard diluent, operator, pipetting and washing technique, incubation time or temperature, and kit age can cause variation in result. The following examples are for the purpose of *illustration only*, and should not be used to calculate unknowns. Each user should obtain their own standard curve.

**EXAMPLE**

The following data was obtained for a standard curve using Calibrator Diluent I.

Standard (pg/mL)	O.D. (450 nm)	Mean	Zero Standard Subtracted (Std.) - (S1)
0	0.082, 0.082	0.082	0
15.625	0.162, 0.161	0.161	0.079
31.25	0.227, 0.222	0.224	0.142
62.5	0.343, 0.357	0.350	0.268
125	0.570, 0.574	0.572	0.490
250	1.009, 1.014	1.011	0.929
500	1.800, 1.814	1.807	1.725
1,000	2.767, 2.834	2.800	2.718



## PERFORMANCE CHARACTERISTICS

### 1. INTRA-ASSAY PRECISION

To determine within-run precision, three different samples of known concentration were assayed by replicates of twenty in 1 assay.

Sample	Calibrator Diluent I Assay		
	1	2	3
n	20	20	20
Mean (pg/mL)	66.35	142.14	538.68
Standard Deviation (pg/mL)	2.29	4.02	13.36
Coefficient of Variation (%)	3.45	2.83	2.48

### 2. INTER-ASSAY PRECISION

To determine between-run precision, three different samples of known concentration were assayed by replicates on 20 different assays.

Sample	Calibrator Diluent I Assay		
	1	2	3
n	20	20	20
Mean (pg/mL)	64.2	141.5	509.7
Standard Deviation (pg/mL)	8	16	17
Coefficient of Variation (%)	12.4	11.2	3.31

### 3. RECOVERY

The recovery of TNF- $\alpha$  spiked to seven different levels in five test samples throughout the range of the assay were evaluated. All samples were mixed and assayed in duplicate.

Sample Type	Average Recovery (%)	Range (%)
Cell Culture Media	102.7	96.0-108.0
Serum	98.5	82.0-128.0
EDTA plasma	109.8	84.0-120.0
Heparin plasma	101.4	85.0-128.0
Citrate plasma	96.6	82.0-128.0

### 4. SENSITIVITY

The minimum detectable dose of TNF- $\alpha$  using a standard curve generated with Calibrator Diluent I or Calibrator Diluent II is 2.4 pg/mL.

### 5. SPECIFICITY

This sandwich ELISA recognises both natural and recombinant human TNF- $\alpha$ . This kit exhibits no significant cross-reactivity with human TGF, MCP-1, MCP-3, M-CSF, GM-CSF, EGF, IL-1 $\beta$ , IL-8, and IL-16.

## 6. CALIBRATION

This immunoassay is calibrated against Ball-1 cell derived human TNF- $\alpha$  (NIBSC/WHO First International Standard 88/786).

## 7. TESTING OF CELL CULTURE SUPERNATANTS

<i>Activation Condition</i>	<i>TNF-<math>\alpha</math> Concentration (pg/mL)</i>
Monocyte supernate, 1% BSA	30
Monocyte supernate, 1% BSA, 100 ng/mL LPS	4950
Monocyte supernate, 5% FBS	0
Monocyte supernate, 5%, FBS, 100 ng/mL LPS	850

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