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Sunlong Medical™ Human/Cynomolgus Monkey TNF-alpha One-Step ELISA Kit

Catalog Number: One-EL232Hu
Size: 96 Test

Storage: 2-8℃/-20℃
Validity Period: 2-8℃: 6 months; -20℃: 12 months

Sensitivity: 1.1 pg/mL
Assay range: 3.9-250 pg/mL

For the quantitative detection of human/cynomolgus monkey TNF-alpha concentrations in serum, plasma and cell

This package insert must be read entirely before using this product. For proper performance, follow the protocol provided with each individual kit.

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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ASSAY PROCEDURE SUMMARY

1. Preset the layout of the microplate, including control group, standard group and sample group, take out the required number of microplate strips and return excess strips to the foil pouch containing the drying reagent pack and reseal; store at 4°C immediately. Microplate strips should be used in one week.

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2. Add 50 µL standard or sample to appropriate wells. To avoid high background always add samples or standards to the well before the addition of antibody cocktail.

↓

3. Add 50 µL 1× Antibody Cocktail solution (refer to Reagent Preparation 7.2) to each well. Seal plate with cover seal and incubate at 37℃ on a microplate thermostatic shaker set at 400 rpm for 1 hour (incubate at 37℃ for 2 hours is recommended if thermostatic shaker is not available).

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4. Wash: 1) Gently remove the cover seal. Discard the liquid from wells by aspirating or decanting. Remove any residual solution by tapping the plate a few times on fresh paper towels. 2) Wash 4 times with 1× Wash Buffer, using at least 350-400 µL per well. Following the last wash, firmly tap plates on fresh towels 10 times to remove residual Wash Buffer. Avoid getting any towel fibers in the wells or wells drying out completely.

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5. Add 100 µL TMB substrate solution to each well, protected from light. Incubate at 37°C on a microplate thermostatic shaker set at 400 rpm for 15 to 20 minutes. (Substrate Solution should remain colorless until added to the plate.)

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6. Add 100 µL Stop Solution to each well in the same order as addition of the TMB substrate. Note: Avoid skin and eye contact with the Stop solution.

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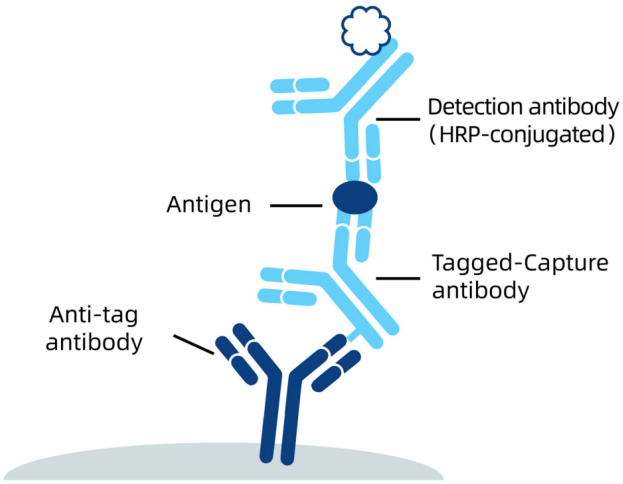
7. Read results immediately on a microplate reader at a wavelength of 450 nm. If possible, perform a double wavelength readout (450 nm and 630 nm).

DESCRIPTION

TNF, as also known as TNF-alpha, or cachectin, is a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. It is expressed as a 26 kDa membrane bound protein and is then cleaved by TNF-alpha converting enzyme (TACE) to release the soluble 17 kDa monomer, which forms homotrimers in circulation. It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons. It can bind to, and thus functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. Dysregulation of TNF production has been implicated in a variety of human diseases including Alzheimer's disease, cancer, major depression and inflammatory bowel disease (IBD).

PRINCIPLE OF THE ASSAY

An anti-tag antibody is pre-coated onto the bottom of wells. After adding antigen or samples, Tagged-Capture antibody and HRP-conjugated detection antibody, a sandwich complex is formed in the solution. TMB acts as a HRP substrate, and the solution color will change from colorless to blue. A stop solution containing sulfuric acid turns the solution yellow. The color intensity is proportional to the quantity of bound protein, which is measurable at 450 nm with the correction wavelength set at 630 nm.



LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use expired kit or reagents.
- Do not mix or substitute reagents or materials from other kit lots or other sources.
- If concentration of assayed factor in samples is higher than the highest standard, dilute the samples with the appropriate Sample Diluent and reanalyze.
- Any variation in testing personnel, sample preparation, standard dilution, pipetting technique, washing techniques, incubation time, temperature, kit age and equipment can cause variation in results.
- This assay is designed to eliminate interference by factors present in biological samples. Until all factors have been tested in the ELISA immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED (96 Test)

Unopened kit should be stored at 2 - 8℃.

- **Microplate:** 96 well microplate precoated an anti-tag antibody(8 well × 12 strips) (1 plate)
- **Protein standard:** 500 pg/bottle; lyophilized (2 bottles)
- **Capture antibody (100X):** 60 µL/vial* (1 vial)
- **Detection antibody, HRP-conjugated (100X):** 60 µL/vial* (1 vial)
- **Sample Diluent PT 3:** 30 mL/bottle. For human serum and plasma. (1 bottle)
- **Sample Diluent PT 1-ef:** 30 mL/bottle. For cell culture supernatant. (1 bottle)
- **Detection Diluent:** 15 mL/bottle (1 bottle)
- **Wash Buffer Concentrate (20×):** 30 mL/bottle (1 bottle)
- **Tetramethylbenzidine Substrate (TMB):** 12 mL/bottle (1 bottle)
- **Stop Solution:** 12 mL/bottle (1 bottle)
- Plate Cover Seals (4 pieces)

STORAGE

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2 to 8°C). Expiration date of the kit and reagents is stated on the labels.

Expiration date of the kit components can only be guaranteed if the components are stored properly.

	Condition
Unopened kit	Store at 2 - 8°C for 6 months or -20°C for 12 months. (See expiration date on the label.)
Opened kit	All reagents stored at 2 - 8°C for 7 days. Please use a new standard for each assay.

OTHER SUPPLIES REQUIRED

- A microplate reader capable of measuring absorbance at 450 nm with the correction wavelength set at 630 nm.
- Calibrated, adjustable precision pipettes and disposable plastic tips. A manifold multi-channel pipette is recommended for large assays.
- Plate washer: automated or manual.
- Absorbent paper towels.
- Glass or plastic tubes to prepare standard and sample dilutions.
- Beakers and graduated cylinders.
- Log-log or semi-log graph paper or computer and software for ELISA data analysis. A four-parameter logistic (4-PL) curve-fit is recommended.
- Microplate thermostatic shaker.

PRECAUTION

- Avoid any skin and eye contact with Stop Solution and TMB. In case of contact, wash thoroughly with water.
- Do not use the kit after the expiration date.
- Do not mix or substitute reagents or materials from other kit lots or other sources.
- Be sure to wear protective equipment such as gloves, masks and goggles during the experiment.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer to improve assay precision

TECHNICAL HINTS

- Bring all reagents to room temperature before use.
- 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.

- When using an automated plate washer, adding a 30 second soak period before washing step and/or rotating the plate between wash steps may improve assay precision.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless 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.
- It is recommended that all samples and standards be assayed in duplicate.

SAMPLE COLLECTION AND STORAGE

Serum

Allow blood samples to clot for 30 minutes, followed by centrifugation for 15 minutes at 1000xg. Clear serum can be assayed immediately or aliquoted and stored at -20℃. Avoid repeated freeze-thaw cycles.

Plasma

Use EDTA, heparin, or citrate as an anticoagulant for plasma collection. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. The plasma can be assayed immediately or aliquoted and stored at -20℃. Avoid repeated freeze-thaw cycles.

Cell Culture Supernatant

Remove particulates by centrifugation for 5 minutes at 500xg and assay immediately or aliquot and store samples at ≤ -20℃. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

Bring all reagents and samples to room temperature before use. If crystals have formed in the Wash Buffer Concentrate, warm to room temperature and mix gently until the crystals have completely dissolved.

Wash Buffer (1X)

If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 30 mL of Wash Buffer Concentrate(20X) to 570 mL deionized or distilled water to prepare 1X Wash Buffer.

Antibody Cocktail (1X)

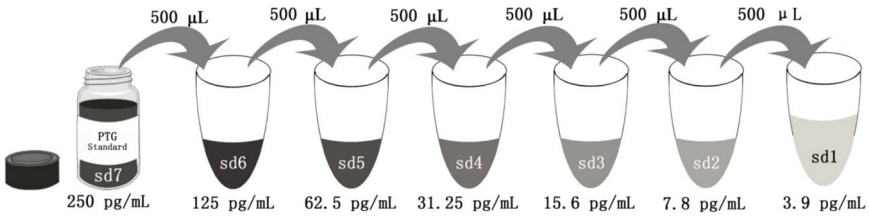
Dilute 100X capture antibody and 100X HRP-conjugated detection antibody using Detection Diluent prior to assay. Suggested 1:100 dilution: 50 µL 100X capture antibody + 50 µL 100X Detection Antibody, HRP-conjugated + 4,900 µL Detection Diluent. Mix gently but thoroughly.

Sample Dilution

Different samples should be diluted with corresponding Sample Diluent. The minimum required dilution factor was 1:2; consequently, no more than 25 µL of undiluted sample was added per well. Samples may require further dilution if the readout values are higher than the highest standard OD reading. Variations in sample collection, processing and storage may affect the results of the measurement. Recommended Dilution for different sample types: 1:2 is recommended for human serum and plasma; 1:2 to 1:80 is recommended for cell culture supernatant.

Standard Serial Dilution

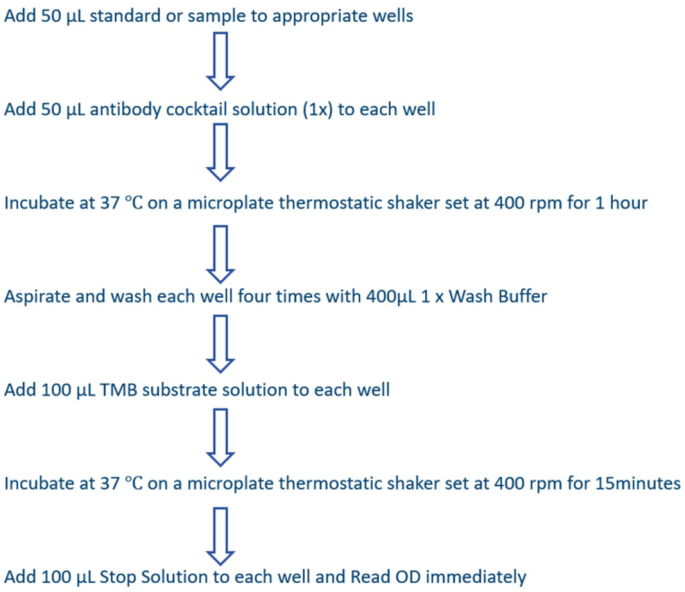
For human serum and plasma, add 2mL Sample Diluent PT 3 in protein standard. For cell culture supernatant, add 2mL Sample Diluent PT 1-ef in protein standard.



Add # µL of Standard diluted in the previous step	—	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL
# µL of Sample Diluent PT 3 or PT 1-ef	**2000 µL**	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL
	"sd7"	"sd6"	"sd5"	"sd4"	"sd3"	"sd2"	"sd1"

ASSAY PROCEDURE

Bring all reagents to room temperature before use (Detection antibody, HRP-conjugated antibody can be used immediately). 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.



1. Preset the layout of the microplate, including control group, standard group and sample group, take out the required number of microplate strips and return excess strips to the foil pouch containing the drying reagent pack and reseal; store at 4°C immediately. Microplate strips should be used in one week.

2. Add 50 µL standard or sample to appropriate wells. To avoid high background always add samples or standards to the well before the addition of antibody cocktail.

3. Add 50 µL 1× Antibody Cocktail solution (refer to Reagent Preparation 7.2) to each well. Seal plate with cover seal and incubate at 37℃ on a microplate thermostatic shaker set at 400 rpm for 1 hour (incubate at 37℃ for 2 hours is recommended if thermostatic shaker is not available).

4. Wash: 1) Gently remove the cover seal. Discard the liquid from wells by aspirating or decanting. Remove any residual solution by tapping the plate a few times on fresh paper towels. 2) Wash 4 times

with 1× Wash Buffer, using at least 350-400 µL per well. Following the last wash, firmly tap plates on fresh towels 10 times to remove residual Wash Buffer. Avoid getting any towel fibers in the wells or wells drying out completely.

5. Add 100 µL TMB substrate solution to each well, protected from light. Incubate at 37°C on a microplate thermostatic shaker set at 400 rpm for 15 to 20 minutes. (Substrate Solution should remain colorless until added to the plate.)

6. Add 100 µL Stop Solution to each well in the same order as addition of the TMB substrate. Note: Avoid skin and eye contact with the Stop solution.

7. Read results immediately on a microplate reader at a wavelength of 450 nm. If possible, perform a double wavelength readout (450 nm and 630 nm).

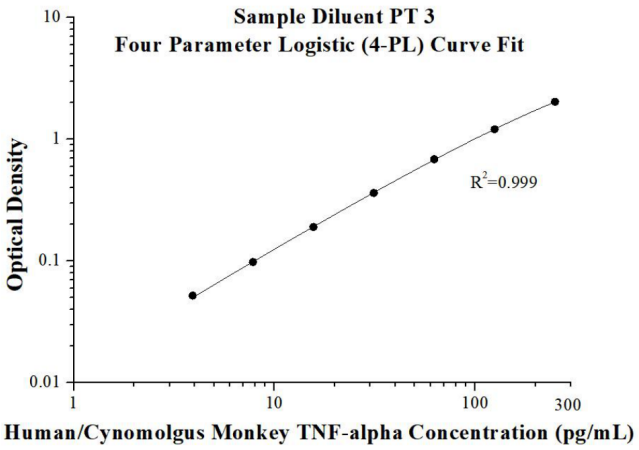
CALCULATION OF RESULTS

Data analysis: Calculate the average of the duplicate readings (OD value) for each standard and sample, and subtract the average of the zero standard absorbance. Construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, four-parameter logistic curvefit (4-PL) analysis is recommend. If the samples have been diluted, the fitting result must be multiplied by the dilution factor used.

TYPICAL DATA

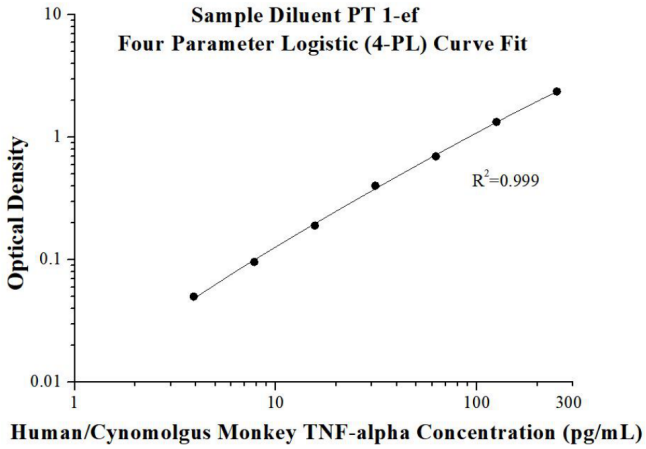
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Standard curve 1



(pg/mL)	O.D.	Average	Corrected
0	0.0345 0.0326	0.03355	-
3.9	0.0855 0.0861	0.0858	0.0523
7.8	0.1332 0.1306	0.1319	0.0984
15.6	0.2273 0.2195	0.2234	0.1899
31.25	0.3981 0.3935	0.3958	0.3623
62.5	0.7237 0.7125	0.7181	0.68455
125	1.2582 1.2285	1.24335	1.2098
250	2.0698 2.0601	2.06495	2.0314

Standard curve 2



(pg/mL)	O.D.	Average	Corrected
0	0.0408 0.0366	0.0387	-
3.9	0.0899 0.0875	0.0887	0.05
7.8	0.1351 0.1333	0.1342	0.0955
15.6	0.231 0.2268	0.2289	0.1902
31.25	0.4408 0.4431	0.44195	0.4033
62.5	0.7418 0.7341	0.738	0.69925
125	1.3619 1.3846	1.37325	1.3346
250	2.3682 2.4506	2.4094	2.3707

SENSITIVITY

The minimum detectable dose of human/cynomolgus monkey TNF-alpha is 1.1 pg/mL. This was determined by adding two standard deviations to the concentration corresponding to the mean O.D. of 20 zero standard replicates.

PRECISION

Intra-assay Precision (Precision within an assay) Three samples of known concentration were tested 8 times on one plate to assess intra-assay precision. Inter-assay Precision (Precision between assays) Three samples of known concentration were tested in 16 separate assays to assess inter-assay precision.

Intra-assay Precision

Sample	n	Mean (pg/mL)	SD	CV%
1	8	128.6	7.6	5.9
2	8	30.3	0.8	2.8
3	8	15.1	0.4	2.5

Inter-assay Precision

Sample	n	Mean (pg/mL)	SD	CV%
1	16	127.7	6.2	4.9
2	16	30.5	1.0	3.3
3	16	15.2	0.5	3.4

RECOVERY

The recovery of human/cynomolgus monkey TNF-alpha spiked to three different levels throughout the range of the assay in various matrices was evaluated.

Sample Type		Average% of Expected	Range (%)
Human serum	1:2	84	76-93
	1:4	91	80-99
Cell culture supernatant	1:100	109	104-112
	1:200	105	100-108

LINEARITY

To assess the linearity of the assay, human serum samples were spiked with high concentrations of human TNF-alpha and diluted with the appropriate Sample Diluent to produce samples with values within the dynamic range of the assay. Cell culture supernatant samples were diluted with the appropriate Sample Diluent to produce samples with values within the dynamic range of the assay. (The human cell culture supernatant was initially diluted 1:40. The cynomolgus monkey cell culture supernatant was initially diluted 1:10.)

		Human serum (Sample Diluent PT 3)	Human cell culture supernatant (Sample Diluent PT 1-ef)	Cynomolgus monkey cell culture supernatant (Sample Diluent PT 1-ef)
1:2	Average% of Expected	89	100	100
	Range (%)	85-94	-	-
1:4	Average% of Expected	102	101	100
	Range (%)	101-103	99-103	99-101
1:8	Average% of Expected	105	102	94
	Range (%)	100-109	101-103	84-103
1:16	Average% of Expected	118	107	100
	Range (%)	109-127	102-113	95-104

CALIBRATION

The NIBSC/WHO Human TNF-alpha Reference Reagent (12/154) (rDNA derived), which was intended as a potency standard, was evaluated in this kit. The dose response curve of this Reference Reagent parallels the standard curve. To convert sample values obtained with the Human TNF-alpha ELISA kit to approximate NIBSC/WHO (12/154) values, use the equation below. NIBSC (12/154) approximate value (IU/mL)=0.04× Human TNF-alpha value (pg/mL)

SAMPLE VALUES

Human serum - human serum samples were evaluated for the presence of TNF-alpha in this assay. ND*=Non-detectable Cell culture supernatant: Human peripheral blood mononuclear cells (PBMC) (1 x 106 cells/mL) were cultured in DMEM supplemented with 10% fetal bovine serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 3 days and 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of TNF-alpha. Cynomolgus monkey peripheral blood mononuclear cells (1 x 106 cells/mL) were cultured in DMEM supplemented 10% fetal bovine serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin and 100µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 50 ng/mL PMA and 1 µg/mL

calcium Ionomycin for 1 day. Aliquots of the cell culture supernates were removed and assayed for levels of TNF-alpha.

Sample Type	Mean (pg/mL)	% Detectable	Range (pg/mL)
Human serum (n=8)	6.3	50	ND-16.1

Condition	Day 3 (pg/mL)	Day 5 (pg/mL)
Unstimulated	13.1	12.3
Stimulated	12,107.3	11,366.0

Condition	Day 1 (pg/mL)
Unstimulated	496.4
Stimulated	5,203.6

SPECIFICITY

This assay recognizes natural and recombinant human/cynomolgus monkey TNF-alpha. The following factors prepared at 50 ng/mL were assayed and exhibited no cross-reactivity or interference. Recombinant human: Recombinant mouse: Recombinant rat: TNF-β TNF-α TNF-α TNF RII TNFSF13

PLATE LAYOUT

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												