INSTRUCTIONS

Human TNFα ELISA Kit

EH3TNFA EH3TNFA2 EH3TNFA5

1337.8

Number	Description
EH3TNFA	**Human Tumor Necrosis Factor α (TNFα) ELISA Kit,** sufficient reagents for 96 determinations
EH3TNFA2	**Human Tumor Necrosis Factor α (TNFα) ELISA Kit,** sufficient reagents for 2 × 96 determinations
EH3TNFA5	**Human TNFα ELISA Kit,** sufficient reagents for 5 × 96 determinations

Kit Contents	EH3TNFA	EH3TNFA2	EH3TNFA5
Anti-human TNFα Precoated 96-well Strip Plate	1 each	2 each	5 each
Lyophilized Recombinant Human TNFα Standard	2 vials	4 vials	10 vials
Sample Diluent, contains 0.1% sodium azide	26mL	2 × 26mL	5 × 26mL
30X Wash Buffer	50mL	2 × 50mL	5 × 50mL
Biotinylated Antibody Reagent, contains 0.1% sodium azide	13mL	2 × 13mL	5 × 13mL
Streptavidin-HRP Reagent	14mL	2 × 14mL	5 × 14mL
TMB Substrate	13mL	2 × 13mL	5 × 13mL
Stop Solution, contains 0.16M sulfuric acid	13mL	2 × 13mL	5 × 13mL
Adhesive plate sealers	6 each	12 each	30 each

For research use only. Not for use in diagnostic procedures.

Storage: For maximum stability, store in a non-defrosting -20ºC freezer and refer to the expiration date for frozen storage on the label. Alternatively, store at 2-8ºC and refer to the expiration date for refrigerated storage. Once thawed, store at 4ºC until the expiration date for refrigerated storage. Kit is shipped on dry ice.

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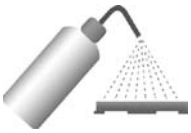
Introduction

The Thermo Scientific™ Human TNFα ELISA is an enzyme-linked immunosorbent assay for measuring human TNFα in serum; EDTA, heparin and sodium citrate plasma; and culture supernatants.

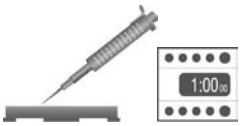
Procedure Summary

1. Add 50µL of Sample Diluent to wells. Add 50µL of Standards or Samples to wells in duplicate.

2. Cover plate and incubate at room temperature (RT) for 1 hour.



3. Wash plate THREE times.

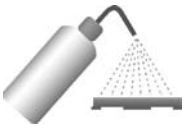


4. Add 100µL of Biotinylated Antibody Reagent to wells. Cover plate and incubate at RT for 1 hour.

5. Wash plate THREE times.

6. Add 100µL of Streptavidin-HRP Reagent to each well.

7. Cover and incubate plate at RT for 30 minutes.



8. Wash plate THREE times.

9. Add 100µL TMB Substrate to each well.

10. Develop plate at room temperature in the dark for 30 minutes.

11. Add100µL of Stop Solution to each well.

12. Measure absorbance and calculate results.

Additional Materials Required

- Precision pipettors with disposable plastic tips to deliver 5-1000µL and plastic pipettes to deliver 5-15mL
- Ultrapure water for Wash Buffer and Standard reconstitution
- A glass or plastic 2L container to prepare Wash Buffer
- A squirt wash bottle or an automated 96-well plate washer
- 1.5mL polypropylene or polyethylene tubes to prepare standards – do not use polystyrene, polycarbonate or glass tubes
- Disposable reagent reservoirs
- A standard ELISA reader for measuring absorbance at 450nm and 550nm. If a 550nm filter is not available, the absorbance may be read at 450nm only. Refer to the instruction manual supplied with the instrument being used.
- Graph paper or a computerized curve-fitting statistical software package

Precautions

- All specimens and reagents must be at room temperature (20-25°C) before use in the assay.
- Review all instructions carefully and verify components against the Kit Contents list (page 1) before beginning the assay.
- Do not use a 37°C water bath to thaw samples. Thaw samples at room temperature.
- If using a multichannel pipettor, always use a new disposable reagent reservoir for the addition of each reagent. Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Use a new adhesive plate cover for each incubation step.
- Avoid microbial contamination of reagents.
- Avoid exposing reagents to excessive heat or light during storage and incubation.
- Do not mix reagents from different kit lots. Discard unused ELISA components after completing the assay.
- Do not use glass pipettes to measure the TMB Substrate Solution. Take care not to contaminate the solution. If the solution is blue before use, DO NOT USE IT.
- Individual components may contain antibiotics and preservatives. Wear gloves while performing the assay to avoid contact with samples and reagents. Please follow proper disposal procedures.
- Some components of this kit contain sodium azide. Please dispose of reagents according to local regulations.

Additional Precautions for the 2-plate and 5-plate Kits

- Dispense, pool and equilibrate to room temperature only the reagent volumes required for the number of plates being used. Do not combine leftover reagents with those reserved for additional plates.

Sample Preparation

- Serum; EDTA, heparin and sodium citrate plasma; and culture supernatant may be tested in this assay; 50µL per well of serum, plasma or culture supernatant is required.
- Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -70°C. Avoid repeated freeze-thaw cycles when storing samples.
- Samples and standards must be assayed in duplicate each time the assay is performed.
- Equilibrate samples gradually to room temperature before beginning the assay. Do not use a heated water bath to thaw or warm samples.
- Mix samples by gently inverting the tubes.
- If samples are clotted, grossly hemolyzed, lipemic or contaminated, make a note on the template and interpret results with caution.
- If the TNFα concentration of a sample possibly exceeds the highest point of the standard curve (i.e., 1000pg/mL), prepare one or more 5-fold dilutions of the sample. Prepare a 5-fold dilution by adding 50µL of sample to 200µL of Sample Diluent and mix thoroughly. Prepare all sample dilutions using the Sample Diluent provided.

Reagent Preparation

For procedural differences when running partial plates, look for **(PP)** throughout these instructions.

Wash Buffer

Note: Wash Buffer must be at room temperature before use. Do not use Wash Buffer if it becomes visibly contaminated.

1. Label a clean glass or plastic 2L container “Wash Buffer.” The 30X Wash Buffer may have a cloudy appearance.
2. Add the entire contents of the 30X Wash Buffer (50mL) bottle to the container and dilute to a final volume of 1.5L with ultrapure water. Mix thoroughly.

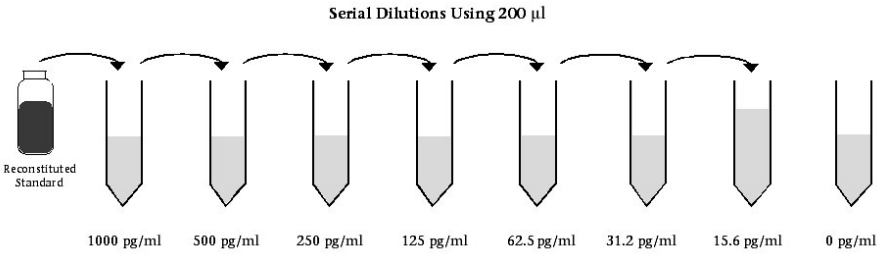
   (PP) When using partial plates, store the reconstituted Wash Buffer at 2-8°C.

Standards

- **(PP)** Reconstitute and use one vial of the lyophilized Standard per partial plate.
- Prepare Standards just before use and use within the same day as reconstitution. Do not store reconstituted standards.
- Reconstitute Standard in ultrapure water. The reconstitution volume is stated on the standard vial label. Mix by gently inverting the vial until the contents have completely dissolved.
- When testing both **serum and cell culture supernatant samples** on the same plate, validate the culture media to establish if the same standard curve can be used for both sample types. Prepare a standard curve (including a zero/blank) using culture medium to reconstitute and dilute the standard. Perform this curve in parallel with a standard curve reconstituted in ultrapure water and diluted in the Sample Diluent provided. If OD values are within 10% of the mean for both curves, then either curve may be used.

1. Label seven tubes, one for each standard curve point: 1000; 500; 250; 125; 62.5; 31.2; 15.6; and 0pg/mL. Prepare 1:2 serial dilutions to generate the standard curve points as follows:
2. Pipette 200µL of Sample Diluent into each tube.
3. Pipette 200µL of the reconstituted Standard into the first tube (1000pg/mL) and mix.
4. Pipette 200µL of this dilution into the next tube (500pg/mL) and mix.

5. Repeat serial dilutions (using 200µL) five more times to complete the standard curve points.



Assay Procedure

A. Sample Incubation

- **(PP)** Determine the number of strips required and leave these strips in the plate frame. Tightly seal the remaining unused strips in the foil pouch with the desiccant provided and store at 2-8°C. After completing the assay, retain the plate frame for the second partial plate. When using the second partial plate, place the reserved strips securely in the plate frame.
- Use the Data Template provided to record locations of standards and test samples. Seven standards and one zero must be assayed in duplicate with each series of unknown samples.
- If the TNFα concentration in any test sample is expected to exceed the highest point on the standard curve (1000pg/mL) refer to the Sample Dilution section.

1. Add 50µL Sample Diluent to each well.
2. Add 50µL standard or sample to each well in duplicate. Mix well by gently tapping the plate several times.
3. Carefully cover plate with an adhesive plate cover. Ensure that all edges and strips are sealed tightly by running your thumb over the edges and down each strip. Incubate for one (1) hour at room temperature, 20-25°C.
4. Carefully remove the adhesive plate cover and wash plate as described in the Plate Washing Section below.

B. Plate Washing

1. Gently squeeze the long sides of plate frame before washing to ensure all strips securely remain in the frame.
2. Empty plate contents. Use a squirt bottle to vigorously fill each well completely with Wash Buffer, then empty plate contents. Repeat procedure two additional times for a total of THREE washes. Blot plate onto paper towels or other absorbent material.

   Note: For automated washing, aspirate all wells and wash THREE times with Wash Buffer, over-filling wells with Wash Buffer. Blot plate onto paper towels or other absorbent material.

C. Biotinylated Antibody Reagent Incubation

- If using a multichannel pipettor, use a new reagent reservoir to add the Biotinylated Antibody Reagent.
- **(PP)** Remove from the vial only the amount required for the number of strips being used.

1. Add 100µL of the Biotinylated Antibody Reagent to each well.
2. Carefully cover plate with an adhesive plate cover. Ensure all edges and strips are tightly sealed by running your thumb over the edges and down each strip. Incubate for one (1) hour at room temperature, 20-25°C.
3. Carefully remove the adhesive plate cover. Wash plate as described in the Plate Washing Section.

D. Streptavidin-HRP Reagent Incubation

- If using a multichannel pipettor, use new reagent reservoir and pipette tips when adding the Streptavidin-HRP Reagent.
- **(PP)** Remove from the vial only the reagent amount required for the number of strips being used.

1. Add 100µL of Streptavidin-HRP Reagent to each well.

2. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate plate for 30 minutes at room temperature, 20-25°C.
3. Carefully remove the adhesive cover and discard plate contents. Wash plate as described in the Plate Washing section.

E. Substrate Incubation and Stop Step

- Use new disposable reagent reservoirs and pipette tips when adding the TMB Substrate Solution and Stop Solution.
- Dispense from the bottle ONLY the amount required for the number of strips being used, 100µL per well. Do not use a glass pipette to measure the TMB Substrate Solution.
- **(PP)** Do not combine leftover substrate with that reserved for strips or plates not being used. Take care not to contaminate the remaining TMB Substrate Solution.

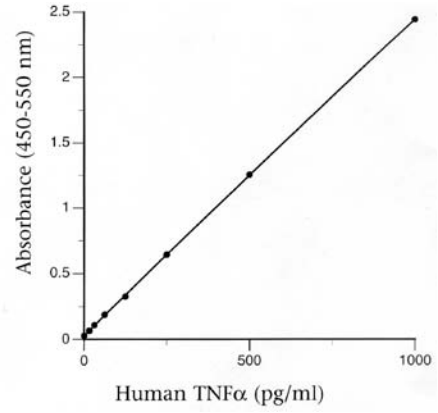
1. Pipette 100µL of TMB Substrate Solution into each well.
2. Allow enzymatic reaction to develop at room temperature in the dark for 30 minutes. **Do not cover plate with aluminum foil or a plate sealer**. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.
3. After 30 minutes, stop the reaction by adding 100µL of Stop Solution to each well.

F. Absorbance Measurement

- The plate must be evaluated within 30 minutes of stopping the reaction. Measure absorbance on an ELISA plate reader set at 450nm and 550nm. Subtract 550nm values from at 450nm values to correct for optical imperfections in the microplate. If 550nm is not available, measure absorbance at 450nm only. Omitting the 550nm measurement will result in higher absorbance values.

G. Calculation of Results

- Generate the standard curve by plotting the average absorbance (450nm minus 550nm) obtained for each Standard concentration on the vertical (Y) axis vs. the corresponding TNFα concentration on the horizontal (X) axis.



- Calculate results manually using graph paper or with a curve-fitting statistical software package. If using curve-fitting software, plot a four-parameter logistic curve fit. Alternatively, a point-to-point curve fit may be used. Determine the amount of TNFα in each sample by interpolating from the TNFα concentration (X axis) to the absorbance value (Y axis).
- If the sample was diluted, multiply the interpolated value obtained by the dilution factor to determine amount of TNFα in the sample.
- Absorbance values obtained for duplicates should be within 10% of the mean value. Carefully consider duplicate values that differ from the mean by greater than 10%.

Performance Characteristics

Sensitivity: < 2pg/mL
The sensitivity or Lower Limit of Detection (LLD)[1] was determined by assaying replicates of zero and the standard curve. The mean signal of zero + 2 standard deviations read in dose from the standard curve is the LLD. This value is the smallest dose that is not zero with 95% confidence.

Assay Range: 15.6-1000pg/mL
Standard curve points are 0, 15.6, 31.2, 62.5, 125, 250, 500 and 1000pg/mL.

Calibration: The standard in this ELISA is calibrated to NIBSC Standard 87/650. One (1) pg = one (1) NIBSC pg = 0.04 units NIBSC units.

Reproducibility: Assay reproducibility was evaluated in each sample matrix. To determine intra-assay precision, 20 replicates of samples containing two levels of recombinant human TNFα were tested on a single plate (Table 1). To evaluate

inter-assay precision, three operators who performed at least three separate assays on more than one day tested samples. Twelve duplicate sample values were used to calculate intra-assay precision data for each level of TNFα (Table 1).

Table 1. Intra- and inter-assay precision of the Thermo Scientific Human TNFα ELISA kit.

		Intra-assay Precision			Inter-assay Precision		
Sample	Level	Mean (pg/mL)	SD (pg/mL)	CV (%)	Mean (pg/mL)	SD (pg/mL)	CV (%)
Serum	1	87.5	3.6	4.2	92.8	4.8	5.2
	2	369	16.6	4.5	384	19.2	5.0
EDTA plasma	1	114	6.7	5.9	117	8.4	7.1
	2	436	21.2	4.8	426	20.2	4.7
Citrate plasma	1	1.5	7.2	6.9	112	9.0	8.0
	2	406	22.2	5.5	384	31.1	7.5
Heparin plasma	1	116	4.1	3.6	116	11.0	9.6
	2	440	17.1	3.9	427	31.0	7.3
Cell culture supernatant	1	170	5.1	3.0	172	7.8	4.6
	2	623	19.7	3.2	622	44.8	7.2

Specificity: The following cytokines, tested at 1 mg/ml, did not interfere with or cross-react in the human TNFα ELISA: human IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12, IL-12p40, IL-13, IL-15, IL-16, IL-17, IL-18, TNFβ, Eotaxin, RANTES, GROα, GROβ, MCP-1, MCP-2, MCP-3, MCP-4, VEGF, GCSF, GMCSF, MIP-1α, MIP-1β, TGFβ, IFNγ, IFNα; mouse TNFα; rat TNFα; and bovine TNFα. TNFα Receptor types 1 and 2 tested at 40mg/mL did not interfere in this assay. Recombinant pig TNFα cross-reacted at 100%.

Expected Values: Thirteen serum and 15 EDTA, heparin and citrate samples collected from apparently healthy individuals were tested. TNFα levels in 50 of the 58 samples tested were below the assay detection limit of 2pg/mL (Table 2).

Table 2. Expected values of TNFα level from healthy individuals.

Sample Type	Mean (pg/mL)	Median (pg/mL)	Range (pg/mL)
Serum (n=35)	< 2	< 2	0-1.3
EDTA plasma (n=35)	2.45	< 2	0-17.3
Citrate plasma (n=8)	< 2	< 2	0-9.6
Heparin plasma (n=13)	2.1	< 2	0-16.6

Recovery: Pooled serum and plasma samples or tissue culture media (TCM) were spiked with recombinant or natural human TNFα. Endogenous TNFα levels were determined by testing non-spiked samples alongside spiked aliquots of the same samples. Expected values were calculated by adding endogenous TNFα levels to those of the spiked control. Percent recovery was calculated by dividing observed by expected values (Table 3).

Table 3. Recovery levels of TNFα from samples spiked with recombinant and natural human TNFα.

	Recombinant TNFα				Natural TNFα			
Sample	n	Mean Expected (pg/mL)	Mean Observed (pg/mL)	Recovery (%)	n	Mean Expected (pg/mL)	Mean Observed (pg/mL)	Recovery (%)
Serum	8	133.2	126.7	95	6	749.1	775.4	104
	8	583.2	562.4	96				
EDTA plasma	8	105.4	91.7	87	6	811.2	722.5	95
	8	561.3	497.8	89				
Citrate plasma	8	119.8	111.7	93	6	751.7	760.7	101
	8	679.5	613.0	90				
Heparin plasma	8	130.1	114.1	88	6	845.9	746.4	88
	8	631.0	597.3	95				
TCM	6	431.5	441.9	102	6	397.0	349.49	88

Dilution Linearity: Serum, plasma or tissue culture media (TCM) were spiked with recombinant or natural human TNFα, serially diluted in Sample Diluent and evaluated. Results for heparin and citrate plasma were similar to those for EDTA plasma. Observed values were compared to expected values to calculate percent recovery and demonstrate the dilution linearity of the assay (Tables 4 and 5).

Table 4. Dilution linearity of samples spiked with recombinant TNFα.

Sample	Dilution	Expected (pg/mL)	Observed (pg/mL)	% Recovery
Serum	Neat	545.6	545.6	-
	1:2	272.8	279.8	103%
	1:4	136.4	146.9	108%
	1:8	68.2	64.1	94%
	1:16	34.1	32.3	95%
EDTA plasma 1	Neat	678.5	678.5	-
	1:2	339.2	354.7	105%
	1:4	169.6	162.0	96%
	1:8	84.8	88.0	104%
	1:16	42.4	40.2	95%
TCM	Neat	415.3	415.3	-
	1:2	207.6	205.5	99%
	1:4	103.8	103.0	99%
	1:8	51.9	46.2	89%
	1:16	26.0	27.3	105%

Table 5. Dilution linearity of samples spiked with natural TNFα.

Sample	Dilution	Expected (pg/mL)	Observed (pg/mL)	% Recovery
Serum 2	Neat	793.6	793.6	-
	1:2	396.8	426.3	107%
	1:4	198.4	198.7	100%
	1:8	99.2	92.1	93%
	1:16	49.6	45.0	91%
EDTA plasma 2	Neat	735.1	735.1	-
	1:2	367.5	386.2	105%
	1:4	183.8	189.3	103%
	1:8	91.9	84.9	92%
	1:16	45.9	45.0	98%
TCM	Neat	356.4	256.4	-
	1:2	178.2	191.7	108%
	1:4	89.1	89.1	100%
	1:8	44.6	39.3	88%
	1:16	22.3	20.3	91%

Cited Reference

1. In *Immunoassay: A Practical Guide*, Chan and Perlstein, Eds. Academic Press: New York, p71.

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Data Templates

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