INSTRUCTIONS

Porcine TNFα ELISA Kit

EP2TNFA EP2TNFA2 EP2TNFA5

1178.5

Number	Description
EP2TNFA	**Porcine Tumor Necrosis Factor alpha (TNFα) ELISA Kit,** sufficient reagents for 96 determinations
EP2TNFA2	**Porcine Tumor Necrosis Factor alpha (TNFα) ELISA Kit,** sufficient reagents for 2 × 96 determinations
EP2TNFA5	**Porcine Tumor Necrosis Factor alpha (TNFα) ELISA Kit,** sufficient reagents for 5 × 96 determinations

Kit Contents	EP2TNFA	EP2TNFA2	EP2TNFA5
Anti-TNFα Coated 96-well Plate	1 each	2 each	5 each
Lyophilized Recombinant Porcine TNFα Standard	2 vials	4 vials	10 vials
Sample Diluent	14mL	2 × 14mL	5 × 14mL
30X Wash Buffer	50mL	2 × 50mL	5 × 50mL
Biotinylated Antibody Reagent	12mL	2 × 12mL	5 × 12mL
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 covers	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™ Porcine TNFα ELISA Kit is for measuring porcine TNFα in serum; EDTA, heparin and sodium citrate plasma; and culture supernatants. This kit is a sandwich ELISA that uses anti-human TNFα antibodies that recognize porcine TNFα. The microplate provided is coated with an antibody that captures TNFα in standards and samples when added to the plate. After non-bound proteins are removed, the biotinylated detecting antibody is added and binds to a second site on the TNFα. The plate is then washed and streptavidin-horseradish peroxidase is added. The enzyme-substrate reaction

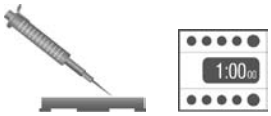
generates a colorimetric signal that is measured on a plate reader at 450nm minus the absorbance at 550nm. The absorbance is proportional to the amount of porcine TNFα in the standard or sample.

Procedure Summary

1. Add 50µL of Standard Diluent to each well. Add 50 µl of standards or samples to each well in duplicate.

2. Cover plate and incubate at room temperature (20-25°C) for 2 hours.

3. Wash plate THREE times.



4. Add 100µL of Biotinylated Antibody Reagent to each well. Cover plate and incubate at room temperature for 1 hour.

5. Wash plate THREE times.

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

7. Cover plate and incubate at room temperature for 30 minutes.

8. Wash plate THREE times.

9. Add 100µL of the TMB Substrate to each well.

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

11. Stop reaction by adding 100µL of Stop Solution to each well.

12. Measure absorbance on a plate reader at 450nm minus 550nm and calculate results.

Additional Materials Required

- Precision pipettors with disposable plastic tips to deliver 5-1000µL and plastic pipettes to deliver 5-15mL
- 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 can be measured 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 samples and reagents must be at room temperature (20-25°C) before use in the ELISA.**
- Review all instructions carefully and verify components against the Kit Contents list (page 1) before beginning the assay.
- Do not use a water bath to thaw samples. Thaw samples at room temperature.
- The Standard Diluent must be used when diluting all samples and standards.
- To avoid cross-contamination use new disposable pipette tips for each transfer and a new adhesive plate cover for each incubation step. If using a multichannel pipettor, always use a new disposable reagent reservoir.
- Once reagents have been added to the plate, take care NOT to let plate DRY at any time during the assay.
- Vigorous plate washing is essential.

- Avoid exposing reagents to excessive heat or light during storage and incubation.
- Do not mix reagents from different kit lots. Discard unused kit components.
- Do not use glass pipettes to measure TMB Substrate. 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 Precaution 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

Sample Handling

- Serum; EDTA, heparin or sodium citrate plasma; or culture supernatants may be tested in this ELISA.
- 50µL per well of standard, serum, plasma or culture supernatant are required.
- Store samples to be assayed within 2-3 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -70°C. Avoid repeat freeze-thaw cycles when storing samples.
- Test samples and standards must be assayed in duplicate each time the ELISA is performed.
- Gradually equilibrate samples to room temperature before beginning assay. Do not use heated water baths to thaw or warm samples. Mix samples by gently inverting tubes.
- If samples are clotted, grossly hemolyzed, lipemic or microbially contaminated, or if there is any question about the integrity of a sample, make a note on the template and interpret results with caution.
- Validate medium when testing culture supernatants and using a medium other than RPMI with up to 20% FCS. To perform this validation, prepare two standard curves as described in the Assay Procedure section, one using culture medium and the other using Sample Diluent. If the mean absorbance values for each point on the two curves are within 10% of, the medium will not interfere with the assay.

Sample Dilution

- If the porcine TNFα concentration possibly exceeds the highest point of the standard curve (i.e., 2000pg/mL), prepare one or more five-fold dilutions of the test sample. For example, a five-fold dilution is prepared by adding 50µL of test sample to 200µL of diluent. When testing **culture supernatants**, prepare the serial dilutions using the culture medium. When testing **serum** or **plasma**, prepare the serial dilutions using the Sample Diluent provided. Mix thoroughly between dilutions before assaying. Prepare all sample dilutions using the Standard Diluent provided.

Reagent Preparation

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

Wash Buffer

1. Label a clean glass or plastic two-liter container “Wash Buffer.” The 30X Wash Buffer may have a cloudy appearance.
2. Add the entire contents of the 30X Wash Buffer bottle (50mL) to the two-liter 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.

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

Standards

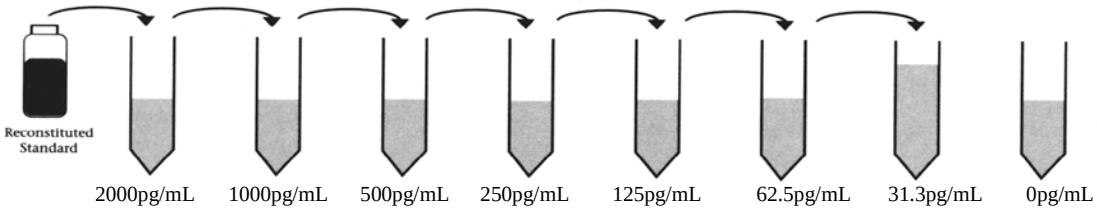
- **(PP)** Reconstitute and use one vial of the lyophilized standard per partial plate.
- Prepare standards just before use and use within one hour of reconstitution. Do not store reconstituted standards.

1. Reconstitute standard with ultrapure water. Reconstitution volume is stated on the standard vial label. Mix by gently inverting the vial until the contents have completely dissolved. Use the Standard Diluent provided to prepare the standard curve serial dilutions.
2. Label eight tubes, one for each standard curve point: 2000pg/mL, 1000pg/mL, 500pg/mL, 250pg/mL, 125pg/mL, 62.5pg/mL, 31.3pg/mL, and 0pg/mL, then prepare 1:2 serial dilutions for the standard curve as follows:
3. Pipette 200µL of diluent into each tube.

   If testing **serum, plasma or culture supernatants** generated with RPMI containing 0-20% FCS, use the Sample Diluent provided to prepare standard dilutions.

   If testing **culture supernatants** generated with other types of media or media containing additives, validate the media to confirm that it will not interfere with the assay. (See Sample Preparation – Sample Handling Section.)
4. Pipette 200µL of the reconstituted standard into the first tube (i.e., 2000pg/mL) and mix.
5. Pipette 200µL of this dilution into the next tube (i.e., 1000pg/mL) and mix.
6. Repeat serial dilutions (using 200µL) five more times to complete the standard curve points.

Serial dilutions using 200µL



Assay Procedure

A. Sample Incubation

- **(PP)** Determine the number of strips required. Leave these strips in the plate frame. Tightly seal remaining unused strips in the provided foil pouch with desiccant and store at 2-8°C. After completing assay, retain plate frame for second partial plate. When using the second partial plate, place strips securely in the plate frame.
- Use the Data Template provided to record the locations of the zero standard (blank or negative control), standards and test samples. Perform seven standard points and one blank in duplicate with each series of unknown samples.

1. Add 50µL of Standard Diluent to each well.
2. Add 50µL of reconstituted standards or test samples in duplicate to each well.

   Note: If the porcine TNFα concentration in any sample possibly exceeds the highest point on the standard curve, 2000pg/mL, see Sample Preparation – Sample Dilution Section.
3. Carefully cover plate with an adhesive plate cover. Ensure all edges and strips are sealed tightly by running your thumb over edges and down each strip. Incubate for two (2) hours at room temperature, 20-25°C.
4. Carefully remove adhesive plate cover and wash plate THREE times with Wash Buffer as described in the Plate Washing Section (section B).

B. Plate Washing

1. Gently squeeze the long sides of plate frame before washing to ensure all strips remain securely in the frame.

2. Discard plate contents. Use a squirt wash bottle to vigorously fill each well completely with Wash Buffer, then discard 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, overfilling 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 and pipette tips** when adding the Biotinylated Antibody Reagent. Remove from the vial only the amount required for the number of strips being used.

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

D. Streptavidin-HRP Solution Incubation

1. Add 100µL of prepared Streptavidin-HRP Solution to wells.
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 plate cover and wash THREE times as described in the Plate Washing Section.

E. Substrate Incubation and Stop Step

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

1. Pipette 100µL of TMB Substrate into each well.
2. Allow enzymatic color reaction to develop at room temperature 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

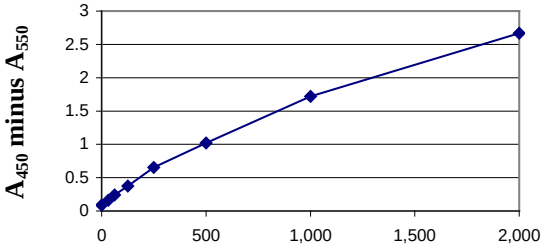
Note: Evaluate the plate within 30 minutes of stopping the reaction.

Measure the absorbance on an ELISA plate reader set at 450nm and 550nm. Subtract 550nm values from 450nm values to correct for optical imperfections in the microplate. If an absorbance at 550nm is not available, measure the absorbance at 450nm only. When the 550nm measurement is omitted, absorbance values will be higher.

G. Calculation of Results

- The standard curve is used to determine porcine TNFα amount in an unknown sample. Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the vertical (Y) axis vs. the corresponding porcine IFNγ concentration (pg/ml) on the horizontal (X) axis.

Standard Curve Example



- Calculate results using a four-parameter logistic curve-fitting software package. If four-parameter is not available, use a point-to-point curve fit. Alternatively, manually graph a point-to-point curve fit using graph paper. Determine the TNFα amount in each sample by interpolating from the absorbance value (Y-axis) to the TNFα concentration (X-axis) using the standard curve.
- If the test sample was diluted, multiply the interpolated value obtained from the standard curve by the dilution factor to calculate pg/ml of porcine TNFα in the sample.
- Absorbance values obtained for duplicates should be within 10% of the mean value. Duplicate values that differ from the mean by greater than 10% should be considered suspect and repeated.

Performance Characteristics

Sensitivity: < 5.0pg/mL
The sensitivity or Lower Limit of Detection (LLD)[1] is 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: 31.3-2000pg/mL
Suggested standard curve points are 2000, 1000, 500, 250, 125, 62.5, 31.3, and 0pg/mL.

Calibration: The standard in this ELISA is calibrated to an internal porcine TNFα reference standard.

Specificity: This ELISA uses antibodies raised against human TNFα. This ELISA reacts with porcine and human TNFα at 100%.

The following cytokines, tested at 1 µg/ml, did not interfere or cross-react: human IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12p70, IL-12p40, IL-13, IL-15, IL-16, IL-17, IL-18, Eotaxin, GCSF, GMCSF, GROα, GROβ, IFNγ, IFNα, MCP-1, MCP-2, MCP-3, MCP-4, VEGF, MIP-1α, MIP-1β, TGFβ, TNFβ or RANTES; mouse TNFα; rat TNFα; bovine TNFα.

The following substances, tested at a concentration of 20µg/mL, did not interfere with this ELISA: Concanavalin A (ConA), phytohemagglutinin (PHA), phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), aprotinin, hemoglobin (HGB), β-mercaptoethanol (β-ME).

Expected Values: A total of eight serum and plasma samples collected from apparently healthy individuals were tested in this ELISA. The levels of porcine TNFα in each sample type are reported in Table 1.

Table 1. Porcine TNFα levels from apparently healthy individuals.

Sample Type	Samples (n)	Samples with DetectableTNFα (>5 pg/mL)	Mean (detectable samples) (pg/mL)	Median (all samples) (pg/mL)	Range (pg/mL)
Serum	8	3	11.7	ND	ND-14.6
EDTA Plasma	8	2	6.0	ND	ND-6.5
Heparin Plasma	8	0	--	ND	ND
Citrate Plasma	8	0	--	ND	ND

ND= Not Detectable

Precision: Reproducibility of the Porcine TNFα ELISA Kit was evaluated in each sample type (Table 2). To determine intra-assay precision, 20 replicates of samples containing either low or high levels of recombinant TNFα were tested on a single plate. To determine inter-assay precision, three different operators tested samples. Each operator performed at least three separate assays on more than one day. Twelve duplicate sample values were used to calculate inter-assay precision.

Table 2. Intra-assay and inter-assay precision of the Porcine 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
Heparin Plasma	1	116	4.1	3.6	116	11.0	9.6
	2	440	17.1	3.9	427	31.0	7.3
Citrate Plasma	1	105	7.2	6.9	112	9.0	8.0
	2	406	22.2	5.5	384	31.1	7.5
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

Spike and Recovery: Porcine serum and plasma samples were spiked with recombinant porcine TNFα. Expected values were calculated by adding endogenous porcine TNFα levels from non-spiked samples to TNFα levels of spiked diluent controls. Percent recovery was calculated by dividing observed by expected values (Table 3).

Table 3. Spike and recovery values from different sample types used in the Porcine TNFα Kit.

Sample	**Samples** (n)	**Expected Value Range** (pg/mL)	**Mean Recovery** (%)	**Median Recovery** (%)	**Recovery Range** (%)
Serum	8	584 - 764	85.2%	89.2%	70.5% - 93.1%
EDTA Plasma	8	590 - 704	96.9%	105.6%	79.0% - 116.3%
Citrate Plasma	8	616 - 699	91.6%	91.0%	79.7% - 100.2%
Heparin Plasma	8	653 - 698	94.5%	97.9%	83.2% - 104.7%

Linearity of Dilution: Eight porcine serum and plasma samples spiked with recombinant porcine TNFα were serially diluted in Sample Diluent and evaluated in this ELISA. Results for a typical evaluation are reported in Table 4. Results for heparin and EDTA plasma were similar to those for citrate plasma. Observed values were divided by expected values to calculate percent recovery and demonstrate dilution linearity.

Table 4. Dilution linearity of the Porcine TNFα ELISA Kit.

Sample	**Dilution**	**Expected Value** (pg/mL)	**Observed Value** (pg/mL)	**% Recovery**
Serum	Neat	449	----	----
	1:2	225	215	96%
	1:4	112	105	93%
	1:8	56	53	95%
	1:16	28	26	92%
Citrate Plasma	Neat	698	----	----
	1:2	349	320	92%
	1:4	174	152	87%
	1:8	87	75	86%
	1:16	44	39	90%

Cited Reference

1. *Immunoassay: A Practical Guide*, Chan and Perlstein, Eds., 1987, Academic Press: New York, p71.

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Data Templates

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