INSTRUCTIONS

Rat TNFα ELISA Kit

ER3TNFA ER3TNFA2 ER3TNFA5

1482.5

Number	Description
ER3TNFA	**Rat Tumor Necrosis Factor Alpha (TNFα) ELISA,** sufficient reagents for 96 determinations
ER3TNFA2	**Rat Tumor Necrosis Factor Alpha (TNFα) ELISA,** sufficient reagents for 2 × 96 determinations
ER3TNFA5	**Rat Tumor Necrosis Factor Alpha (TNFα) ELISA,** sufficient reagents for 5 × 96 determinations

Kit Contents	ER3TNFA	ER3TNFA2	ER3TNFA5
Anti-Rat TNFα Pre-coated 96-well Strip Plate	1 each	2 each	5 each
Lyophilized Recombinant Rat TNFα Standard	2 vials	4 vials	10 vials
Standard Diluent	25mL	2 × 25mL	5 × 25mL
Pre-Treatment Buffer	6mL	2 × 6mL	5 × 6mL
Biotinylated Antibody Reagent	6mL	2 × 6mL	5 × 6mL
Streptavidin-HRP Reagent	14mL	2 × 14mL	5 × 14mL
30X Wash Buffer	50mL	2 × 50mL	5 × 50mL
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.

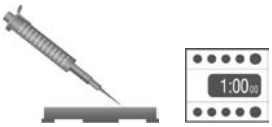
Table of Contents

Introduction

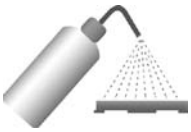
The Thermo Scientific Rat TNFα ELISA Kit is for measuring rat TNFα in serum, EDTA plasma and culture supernatant.

Procedure Summary

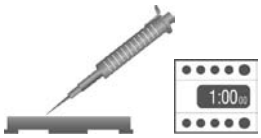
1. Add 50µL of Pre-Treatment Buffer to each well.



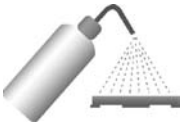
2. Add 50µL of Standards or samples to each well in duplicate.* Cover plate and incubate at room temperature (20-25°C) for 1 hour.



3. Wash plate THREE times.

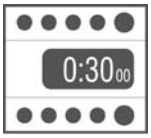


4. Add 50µL of Biotinylated Antibody Reagent to each well. Cover plate and incubate at room temperature for 1 hour.

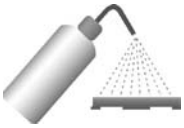


5. Wash plate THREE times.

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

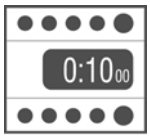


7. Cover and incubate plate at room temperature for 30 minutes.



8. Wash plate THREE times.

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



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

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

12. Measure the absorbance on a plate reader at 450nm minus 550nm. Calculate results.

* Always dilute serum and EDTA plasma samples 1:1 before testing.

Additional Materials Required

- Precision pipettors with disposable plastic tips to deliver 5-l000µL 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 assay.**
- Review these instructions carefully and verify all components against the Kit Contents list (page 1) before beginning.
- Do not use a water bath to thaw samples. Thaw samples at room temperature.
- When assaying culture medium, prepare the standard curve and sample dilutions using the same medium used to culture cells. For example, if RPMI with 10% fetal calf serum (FCS) was used to culture cells, then use RPMI with 10% FCS to dilute standard and samples. For best results, use a culture medium that contains a carrier protein such as FCS. Lack of a carrier protein in the media or addition of other compounds may compromise assay results. If alternative media must be

used, prepare two standard curves: one with Standard Diluent and one with the alternative media. If the two curves have OD values within 10% of the mean for both curves, then perform the assay with Standard Diluent. If the OD values of the two curves are not within 10% of the mean for both curves, then perform the assay with the alternative media. If the alternative media has significant effects on the assay, consider using different media.

- To avoid cross-contamination, always use a new disposable reagent reservoir and new disposable pipette tips for each transfer. Also use a new adhesive plate cover for each incubation step.
- Once reagents have been added to the plate, DO NOT let the plate DRY at any time during the assay.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can become contaminated thereby causing assay variability.
- Vigorous plate washing is essential.
- Avoid exposing reagents to excessive heat or light during storage and incubation.
- When completed with kit, discard unused ELISA components. Do not mix reagents from different kit lots.
- Do not use glass pipettes to measure 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.

Additional Precautions for the 2- 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 plasma and culture supernatants may be tested in this assay.
- For each well, 50µL of EDTA plasma, culture supernatant or 1:1 diluted serum is required.
- Store samples to be assayed within 2 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -70°C.
- Avoid repeated freeze-thaw cycles when storing samples.
- Test samples and standards must be assayed in duplicate each time the assay is performed.
- Gradually equilibrate samples to room temperature before beginning assay. Do not use a heated water bath 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.

Sample Dilution

- **Serum and EDTA plasma samples must first be diluted 1:1 before testing.** To prepare a 1:1 dilution, add 100µL of sample to 100µL of Standard Diluent in a separate tube and mix well. Alternatively, after adding the Pre-Treatment Buffer to the wells add 25µL of Standard Diluent to the appropriate sample wells and then add 25µL of sample. Tap the plate gently to mix. Either method produces a final 1:1 dilution of the sample in each well.
- If the rat TNFα concentration possibly exceeds the highest point of the standard curve (i.e., 2500pg/mL), prepare one or more five-fold dilutions of the test sample. When testing **culture supernatants**, prepare the serial dilutions using culture medium (see Precautions Section). When testing **serum or plasma**, prepare the serial dilution using the Standard Diluent provided. For example, prepare a five-fold dilution by adding 50µL of test sample to 200µL of appropriate diluent. Mix thoroughly between dilutions before assaying.

Reagent Preparation

For procedural differences when using partial plates, look for **(PP)** throughout this instruction booklet.

Wash Buffer

- **(PP)** When using partial plates, store the Wash Buffer at 2-8°C.
- **Note:** Wash Buffer must be at room temperature before use in the assay. Do not use Wash Buffer that has become visibly contaminated during storage.

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 2L container and dilute to a final volume of 1.5L with ultrapure water. Mix thoroughly.

Standards

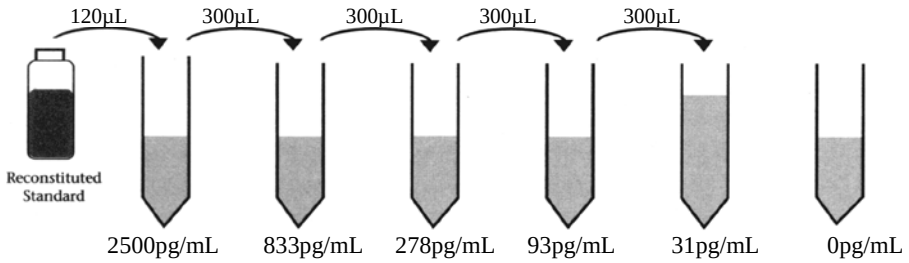
- **(PP)** Reconstitute and use one vial of the lyophilized Standard per partial plate.
- Prepare Standards just before use and use within one hour of reconstitution. Do not store reconstituted standards.

1. For **culture supernatant** samples, reconstitute standard with ultrapure water to the volume indicated on the standard vial label. The standard will dissolve in approximately 1 minute. Mix by gently inverting vial. Use the sample culture medium to prepare Standard Curve dilutions (see Precautions Section).

   For **serum** or **plasma** samples, reconstitute standard with ultrapure water to the volume indicated on the standard vial label. The standard will dissolve in approximately 1 minute. Mix by gently inverting vial. Use the Standard Diluent provided to prepare standard curve serial dilutions.

   When testing **serum** or **plasma** and **cell culture supernatant** samples on the same plate, validate the media to establish if the same standard curve can be used for both sample types. Prepare a standard curve (including a zero) using culture medium to dilute the standard. Use medium containing serum or other protein to maximize stability of the rat TNFα. Perform this curve in parallel with a standard curve prepared with Standard Diluent. If the OD values of the two curves are within 10% of the mean for both curves, then perform the assay with Standard Diluent, whether testing culture supernatant, plasma or serum samples.
2. Label six tubes, one for each standard curve point: 2500, 833, 278, 93, 31, and 0pg/mL. Prepare an initial 1:6 dilution followed by 1:3 serial dilutions for the standard curve as follows:
3. Pipette 600µL of appropriate diluent into each tube.
4. Pipette 120µL of the reconstituted standard into the first tube (i.e., 2500pg/mL) and mix.
5. Pipette 300µL of this dilution into the second tube (i.e., 833pg/mL) and mix.
6. Repeat serial dilutions (using 300µL) three more times to complete the standard curve points.

Standard Dilutions Schematic



Assay Procedure

A. Sample Incubation

- **(PP)** Determine the number of strips required. 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 the zero standard, rat TNFα standards and test samples. Perform five standard points and one blank in duplicate with each series of unknown samples.

1. Add 50µL of the Pre-Treatment buffer to each well.
2. Add 50µL of the reconstituted standard or diluted sample to each well in duplicate. Mix by gently tapping the plate several times.

   Note: All serum and EDTA plasma samples must be diluted 1:1 before testing (see the Sample Dilution Section). If the TNFα concentration in any test sample is expected to exceed the highest point on the standard curve (i.e., 2500pg/mL) refer to the Sample Dilution section.
3. Add 50µL of Standard Diluent to all wells that do not contain standards or samples.
4. 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 (i.e., 20-25°C).
5. Carefully remove the adhesive plate cover. Wash plate THREE times with Wash Buffer using the procedure described in the Plate Washing Section (Section B).

B. Plate Washing

Note: Automated plate washers may produce sub-optimal results. For best results, perform a manual wash the first time the assay is performed. Automated plate washing may require validation to determine the number of washes necessary to achieve optimal results; typically, 3-5 washes are sufficient.

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 wash 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 with Wash Buffer, overfilling wells with Wash Buffer. Blot plate onto paper towels or other absorbent material. The number of washes necessary may require optimization.

C. Biotinylated Antibody Reagent Incubation

- If using a multichannel pipettor, **use new reagent reservoir and pipette tips** when adding the Biotinylated Antibody Reagent.

1. Add 50µL of Biotinylated Antibody Reagent to each well.
2. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate plate for 1 hour at room temperature.
3. Carefully remove the adhesive plate cover, discard plate contents and wash THREE times 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.

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.

3. Carefully remove the adhesive plate cover, discard plate contents and wash THREE times as described in the Plate Washing Section (section B).

E. Substrate Incubation and Stop Step

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

1. Pipette 100µL of TMB Substrate Solution into each well.
2. Allow the color reaction to develop at room temperature in the dark for 10 minutes. Do not cover plate with a plate sealer. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.
3. After 10 minutes, stop the reaction by adding 100µL of Stop Solution to each well.

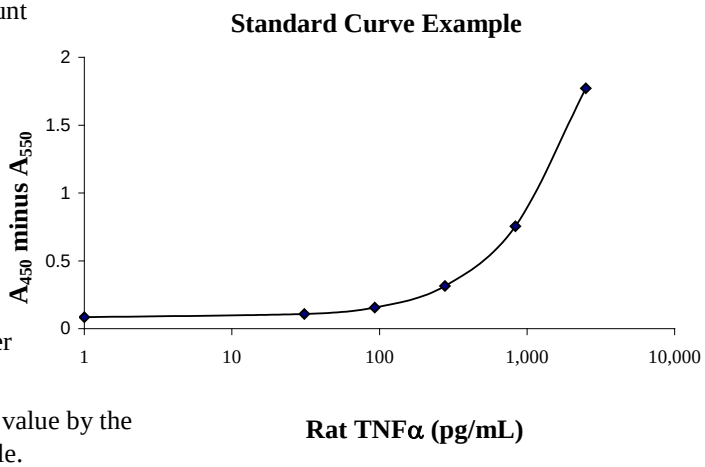
F. Absorbance Measurement

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

Measure 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 rat 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 TNFα concentration (pg/mL) on the horizontal (X) axis.
- Calculate results using graph paper or curve-fitting statistical software. Determine the TNFα amount in each sample by interpolating from the absorbance value (Y-axis) to TNFα concentration (X-axis) using the standard curve. For best results, use a four-parameter curve fit.



- If the test sample was diluted, multiply the interpolated value by the dilution factor to calculate pg/mL 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: < 15pg/mL
The sensitivity or lower limit of detection (LLD)[1] was determined by assaying 24 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.

Assay Range: 31-2500pg/mL
Suggested standard curve points are 2500, 833, 278, 93, 31, and 0pg/mL.

Specificity: The antibodies utilized in this ELISA are specific for the measurement of natural and recombinant rat TNFα. They do not cross-react with human TNFα, human TNFβ or porcine TNFα. Mouse TNFα cross-reacted weakly (< 3%).

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

Precision/Reproducibility:

Intra-assay CV: < 10% (Table 1)
Inter-assay CV: < 10% (Table 2)

Table 1. Intra-assay results using the rat TNFα ELISA kit.

Mean (pg/mL)	%CV	n
2022	3.2	24
912	4.2	24
148	4.0	24

Table 2. Inter-assay results using the rat TNFα ELISA kit.

Mean (pg/mL)	%CV	n
2021	2.9	4
898	3.8	4
142	4.7	4

Recovery: Cytokine recovery was determined by spiking recombinant rat TNFα into serum and plasma samples collected from apparently healthy rats and a Standard Diluent control buffer (Table 3).

Table 3. Spike and recovery results using the rat TNFα ELISA Kit.

	Mean Percent Recovery	
Spike Level	Serum	Plasma
High	102	118
Medium	78	95
Low	75	84
Mean	**85**	**99**
Range	**73-105**	**68-119**

Cited Reference

1. *Immunoassay: A Practical Guide*, ed. Chan and Perlstein, Eds. (1987). Academic Press: New York, p.71.

Data Templates

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

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