

# HUMAN TNF- $\alpha$ (TUMOR NECROSIS FACTOR $\alpha$ ) ELISA KIT

## INSTRUCTION MANUAL (CAT. No. X1851K)

### FEATURES

- Easy to use system
- Reagents titered for success
- Proven protocol



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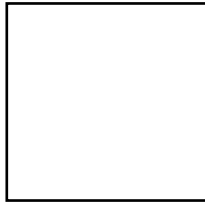
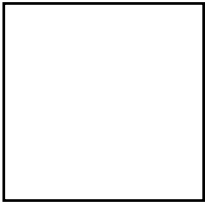
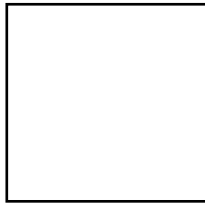
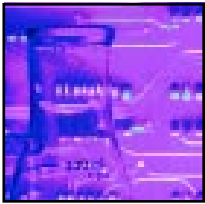
# Other ELISA Kits Available from Exalpha Biologicals

## BROMODEOXYURIDINE PROLIFERATION ASSAY

- Non-radioactive alternative to  $^3\text{H}$ -Thymidine Assay
- Suitable for adherent or non-adherent cell types
- High sensitivity (40 cells/well)
- HTS compatible
- 2.5 hour protocol
- Available in either colorimetric or chemiluminescent

## PIG3 (P53 INDUCIBLE GENE-3) ELISA

- PIG3 is a long-lived reporter of p53 activation
- Assay suitable for sera, plasma, tissue culture supernatants and cellular extracts or lysates
- High sensitivity (40 pg/ml)

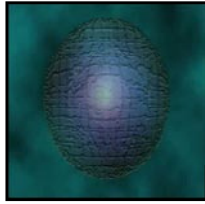
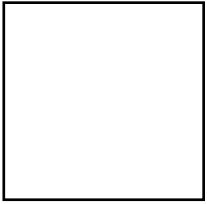


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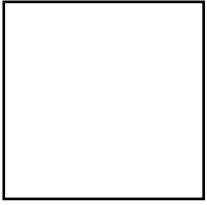
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# HUMAN TUMOR NECROSIS FACTOR $\alpha$ (HUMAN TNF $\alpha$ )



## Intended Use

The Exalpa Biologicals, Inc. human Tumor Necrosis Factor alpha (TNF $\alpha$ ) ELISA is a non-isotopic immunoassay for the in vitro quantitation of human phospho TNF $\alpha$  in serum, plasma, cell culture supernatants.

**This assay is for research use only and not for use in diagnostic or therapeutic procedures.**

## Storage of Kit Components

The Exalpa Biologicals Human TNF $\alpha$  ELISA kit components are shipped on blue ice. Upon receipt, store entire kit at 4-8°C.

## Background

The Exalpa Biologicals, Inc. human Tumor Necrosis Factor alpha (TNF $\alpha$ ) ELISA is a non-isotopic immunoassay for the in vitro quantitation of human TNF $\alpha$  in cell culture supernatants, plasma, or serum. TNF $\alpha$  is a 17Kda protein produced primarily by activated monocytes (macrophages). This polypeptide has been implicated in cell trafficking, inflammatory responses, and defense against pathogens, has both beneficial and deleterious effects. Inhibition of TNF activity may be beneficial in various inflammatory disorders and inhibition of TNF may therefore increase susceptibility to certain pathogens. TNF derived from different cellular sources plays distinct biological roles and suggests that selective inhibition of T cell-derived TNF production may be therapeutically preferable to systemic inhibition of TNF activity. TNF acts as a central mediator in chronic inflammation and is involved in joint swelling, degradation and loss of function in rheumatoid arthritis (RA), has been implicated in cancer invasive phenotypes, involved in insulin resistance (obesity) to name but a few.

## Principles of the Assay

The Exalpa Biologicals, Inc. TNF $\alpha$  ELISA is a non-isotopic immunoassay for the in vitro quantitation of human phospho TNF $\alpha$  protein in cell lysates.

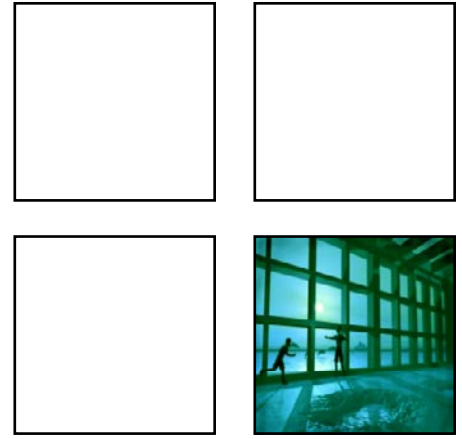
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The TNF $\alpha$  ELISA is a “sandwich” enzyme immunoassay employing polyclonal antibodies. An antibody, specific for human TNF $\alpha$  protein has been immobilized onto the surface of microtiter wells provided in the kit. The sample (serum, plasma, tissue culture supernatant) to be assayed are pipetted into the wells and allowed to incubate for two hours (or over night for higher sensitivity), during which time any TNF $\alpha$  present binds to the capture antibodies. Unbound material is washed away and a biotin conjugated anti- TNF $\alpha$  antibody is added to the wells and incubated for 2 hours at room temperature. Excess biotin conjugate is removed by washing and a horseradish peroxidase (HRP)-conjugated streptavidin is added for 30 minutes, which binds to the detector antibody. Excess HRP conjugate is removed by washing.

The horseradish peroxidase catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) (30 minute incubation) from a colorless solution to a blue solution (or yellow after the addition of stop reagent), the intensity of which is proportional to the amount of TNF $\alpha$  protein in the sample. The colored reaction product is quantified using a spectrophotometer.

Quantitation is achieved by the construction of a standard curve using known concentrations of TNF $\alpha$  standard (provided lyophilized). By comparing the absorbance obtained from a sample containing an unknown amount of TNF $\alpha$  with that obtained from the standards, the concentration of TNF $\alpha$  in the sample can be determined.



## Materials Provided\*

Standards and samples should be assayed in duplicate. A standard curve must be performed on the same plate and at the same time as the samples. The TNF $\alpha$  ELISA provides sufficient reagents to run two sets of standard curves, and 41 samples (if assayed in duplicate all at once using one standard curve), or 34 samples (if assayed on two separate occasions using two standard curves).

- **Component 1:** Coated Microtiter Plate: 96 removable wells coated with anti-TNF $\alpha$  monoclonal antibody.
- **Component 2:** TNF $\alpha$  Standard: two vials containing lyophilized human TNF $\alpha$  protein.  
! Reconstituted standards should be aliquoted and frozen at -30° C and discarded after one freeze thaw.
- **Component 3:** Detector Antibody (12 ml): biotinylated anti-human TNF $\alpha$  antibody.
- **Component 4:** 400X Conjugate (50  $\mu$ l): Streptavidin-Peroxidase Conjugate; 400-fold concentrated solution.
- **Component 5:** Conjugate Diluent (12 ml): buffer for dilution of 400X Conjugate.
- **Component 6:** Substrate (11 ml): chromogenic substrate (TMB).
- **Component 7:** Standard and Sample Diluent (20 ml): buffer used to dilute standards and samples.
- **Component 8:** 50X Plate Wash Concentrate (40 ml): 50-fold concentrated solution of PBS and surfactant.
- **Component 9:** Plate Sealers (2): to cover plates during incubations.

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# Materials Required But Not Provided

- 2-20  $\mu$ l, 20-200  $\mu$ l and 200-1000  $\mu$ l precision pipetters with disposable tips.
- Automated plate washer, wash bottle or multichannel dispenser for washing.
- 2 liter graduated cylinder.
- Deionized or distilled H<sub>2</sub>O.
- Spectrophotometer capable of measuring absorbance in 96-well plates using dual wavelength of 450/595 nm or 450/550 nm. A single wavelength of 450 nm can also be used.

# Summary of Procedure

Not to be used in place of Detailed Assay Protocol. For complete instructions see Detailed Protocol section.

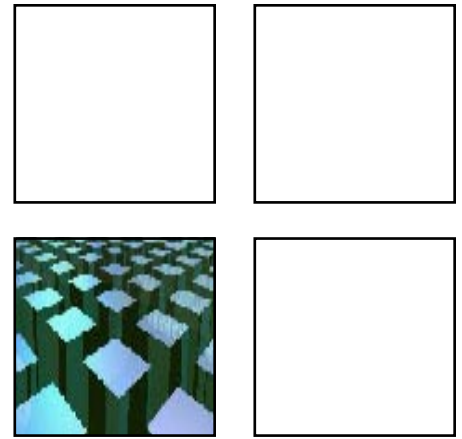
|     | <u>Steps</u>                        | <u>Incubation</u>                     |
|-----|-------------------------------------|---------------------------------------|
| 1.  | Equilibrate kit to room temperature | -                                     |
| 2.  | Add samples and standards to wells  | 2 hours room temp. (or overnight 4°C) |
| 3.  | Wash 4x                             | -                                     |
| 4.  | Add detector antibody to all wells  | 2 hours - room temp.                  |
| 5.  | Wash 4x                             | -                                     |
| 6.  | Add HRP conjugate to all wells      | 30 minutes - room temp.               |
| 7.  | Wash 4x                             | -                                     |
| 8.  | Add substrate to all wells          | 30 minutes - room temp.               |
| 9.  | Add stop solution to all wells      | -                                     |
| 10. | Read plate at 450 nm/550 nm         | -                                     |

# Precautions and Recommendations

- Special care should be taken when working with human serum or plasma samples. Refer to your institutions health and safety guidelines. Wear protective clothing, disposable gloves and eye protection. Use of a BL-2 lab facility is recommended.
- **Store all components at 2°C - 8°C.** Do not expose reagents to excessive heat or light.
- Let the kit sit at room temperature for 30 minutes before use. Best results will be obtained using reagents at room temperature.
- Wear disposable gloves and eye protection and protective clothing as required.
- Do not use the kit beyond the expiration date.
- Always use clean well-rinsed glassware. Soap residue may compromise assay performance.
- Use only the microtiter wells provided with the kit.
- Do not mix reagents from different kits.



- Do not mouth pipette or ingest any of the reagents.
- The buffers and reagents used in this kit contain anti-microbial and anti-fungal reagents. Care should be taken to prevent direct contact with these products.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Do not mouth pipette or ingest any of the reagents.
- The buffers and reagents used in this kit contain anti-microbial and anti-fungal reagents. Care should be taken to prevent direct contact with these products.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- **Human samples may be contaminated with infectious agents. Do not ingest, expose to open wounds, or breathe aerosols. Dispose of samples properly.**



## Sample Preparation

### Specimen Collection and Handling:

Sera and Plasma specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

Cell Culture Supernatant: If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at  $< -20^{\circ}\text{C}$ . Avoid repeated freeze/thaw cycles.

Serum: Use a serum separator tube and allow clotting for 30 minutes, then centrifuge for 10 minutes at  $1000 \times g$ . Remove serum layer and assay immediately or store serum samples at  $< -20^{\circ}\text{C}$ . Avoid repeated freeze/thaw cycles.

Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at  $1000 \times g$  within 30 minutes of collection. Assay immediately or store plasma samples at  $< -20^{\circ}\text{C}$ . Avoid repeated freeze/thaw cycles.

Samples found to contain greater than  $1 \text{ ng/ml TNF}\alpha$  (i.e., outside the range of the standard curve) must be diluted with Sample Diluent (provided), so that the  $\text{TNF}\alpha$  concentration falls within the range spanned by the standard curve, and assayed again.

## Detailed Protocol

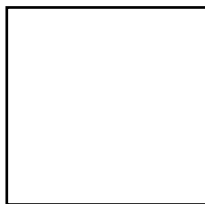
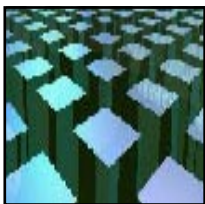
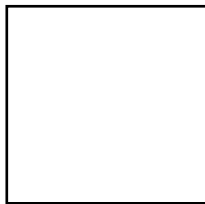
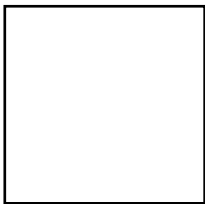
The  $\text{TNF}\alpha$  ELISA is provided with removable strips of wells so the assay can be carried out on two separate occasions. **Since conditions may vary, a standard curve must be determined each time the assay is performed.** Standards should be assayed in duplicate. Disposable pipette tips and reagent troughs should be used for all transfers to avoid cross-contamination of reagents or samples.

1. Remove the appropriate number of microtiter wells from the foil pouch. Return any unused wells to the foil pouch, reseal and store at  $4^{\circ}\text{C}$ . Let all other kit components sit at room temperature until used.

**Best results will be obtained using reagents at room temperature.**

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2. Prepare a working solution (1X) of Wash Buffer by adding 40 ml of the 50X concentrated solution (provided - Component 8), to 1960 ml of deionized water. Mix well.
3. Each time an assay is performed, reconstitute a Lyophilized Standard by carefully and accurately pipetting dH<sub>2</sub>O, as described on the lyophilized TNF $\alpha$  Standard vial label and package insert. Let the reconstituted standard sit for 10 minutes at room temperature, with occasional swirling. Avoid excessive agitation of the standard. After reconstituting the TNF $\alpha$  Standard it should be diluted with Standard Sample Diluent (Component 7). Obtain six tubes and label them 1000, 500, 250, 125, 62.5, 31.25 and 0 pg/ml. Add 250  $\mu$ l of Sample Diluent into each tube except the 1000 pg/ml tube (first tube) which gets “undiluted” reconstituted standard. Remove 500  $\mu$ l from the original vial of lyophilized material and add it to the first tube. Remove 250  $\mu$ l from the first tube (1000 pg/ml) and add it to the second tube (500 pg/ml) and mix gently. Repeat this procedure until you reach the fifth tube (31.25 pg/ml). The last tube (0 ng/ml) should just be Sample Diluent. **Reconstituted standards should be discarded after one use.**
4. Prepare all samples. **A recommended starting dilution for all samples is a 1:4 dilution with Sample and Standard diluent.**
5. Add 50  $\mu$ l of Standard and Sample Diluent to each well to be used.
5. Add samples and each of the TNF $\alpha$  standards (in duplicate) by pipetting 50  $\mu$ l into appropriate wells using clean pipette tips for each sample.
6. Cover wells with a plate sealer and incubate at room temperature for 2 hours (or Overnight at 4° C for higher sensitivity).
7. Remove contents of wells by inverting over sink and tapping on paper towels and wash wells 4 times with 1X Wash Buffer making sure each well is filled completely.
8. Add 100  $\mu$ l of prediluted Detector Antibody (Component 3) to each well used. Cover and incubate for 2 hours at room temperature.
9. Remove contents of wells by inverting over sink and tapping on paper towels and wash wells 4 times with 1X Wash Buffer making sure each well is filled completely.
9. Dilute a sufficient amount of the 400X Conjugate 1:400 in Conjugate Diluent to provide 100  $\mu$ l of 1X solution for each sample and standard well (For example: add 30  $\mu$ l to 11.970 ml of Conjugate Diluent), mix gently.
10. Pipette 100  $\mu$ l of the 1X Conjugate into each well, cover with a plate sealer and incubate at room temperature for 30 minutes. Discard any unused 1X Conjugate.
11. Wash wells 4 times with 1X Wash Buffer making sure each well is filled completely.
12. Remove contents of wells by inverting over sink and tapping on paper towels.
13. Add 100  $\mu$ l of Substrate Solution to each well and incubate **in the dark** at room temperature for 30 minutes.
14. Add 100  $\mu$ l of Stop Solution to each well **in the same order** as the previously added Substrate Solution.
15. Measure absorbance in each well using a spectrophotometric plate reader. It is preferable to read at dual wavelengths of 450/550 nm (or 450/595 nm). A single wavelength of 450 nm can also be used. Wells must be read within 30 minutes of adding the Stop Solution.



# Evaluation of Results

1. Average the duplicate absorbance values for each standard, including the zero, and all sample values.
2. On graph paper, plot the mean absorbance values for each of the standards on the Y axis, versus the concentration of each standard (pg/ml) on the X axis.
3. Determine the concentration of unknowns by interpolation from the standard curve. There are a variety of microtiter plate reader software packages available (Softmax, Molecular Devices Corporation, Menlo Park, CA; KinetiCalc, BioTek Instruments, Inc. Winooski, VT) for analysis of microtiter plate data, which simplifies this process.
4. For samples which have been diluted, the TNF $\alpha$  concentration must be multiplied by the dilution factor (ie., if the sample was diluted five-fold, then the TNF $\alpha$  value obtained from the standard curve must be multiplied by five).

## Assay Characteristics

### Sensitivity

The lower limit of detection (LLD), commonly used to define sensitivity, was measured by assaying four replicates of zero eight times using two different lots of plates and two different lots of detector antibody. The LLD for Exalpha's TNF $\alpha$  assay was determined to be 20 pg/ml.

### Precision

The pooled coefficients of variation (according to the formula of Henry et. al. 1974) and between assay coefficients of variation are plotted against TNF $\alpha$  levels. The pooled data were collected from samples run eight times using two different lots of plates and two different lots of detector antibody in replicates of four on two separate occasions. Precision was determined to be less than 10%.

### Linearity

To assess the linearity of the assay, samples containing various levels of TNF $\alpha$  or spiked with various concentrations of human TNF $\alpha$  were diluted with Sample Diluent and then assayed. The measured human TNF $\alpha$  concentrations at each dilution within the working range of the assay are within 5% of the expected values for all samples.

## Reagent Stability

All of the reagents included with the TNF $\alpha$  have been tested for stability. Reagents should not be used beyond the stated expiration date.

## References

1. Aggarwal, B.B. "Signalling pathways of the TNF superfamily: a double-edged sword." *Nat. Rev. Immunol.* 2003, 3, 745-756
2. Locksley, R.M., et al. "The TNF and TNF receptor superfamilies: integrating mammalian biology." *Cell* 2001, 104, 487-501
3. Taylor, P.C., et al. "Tumour necrosis factor alpha as a therapeutic target for immune-mediated inflammatory diseases." *Curr. Opin. Biotechnol.* 2004, 15, 557-563

## Notes



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## **Ordering Information**

| CATALOG NUMBER | SIZE    |
|----------------|---------|
| X1851K         | 1 PLATE |

## **Contact Information**

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