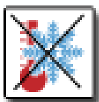
TNFα (rat) ELISA kit

Catalog # ADI-900-086A

96 Well Enzyme Immunoassay Kit

For use with culture supernates



Reagents require separate storage conditions.

Check our website for additional protocols, technical notes, MSDS and FAQs.

For proper performance, use the insert provided with each individual kit received.

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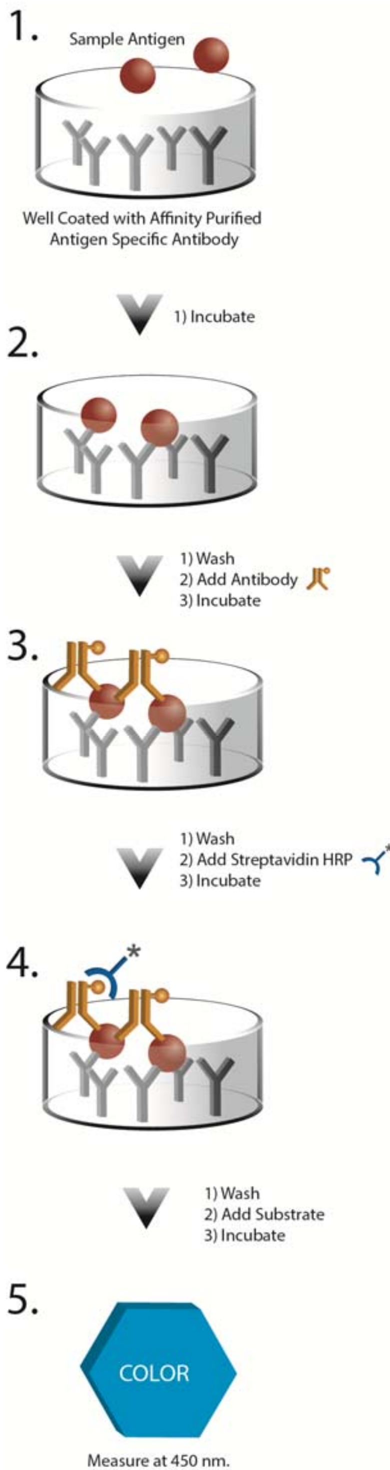


Introduction

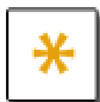
The TNF-α (rat), ELISA kit is a complete kit for the quantitative determination of rat TNF-α in culture supernates. Please read the complete kit insert before performing this assay.

Tumor necrosis factor-alpha (TNF-α), also known as cachectin, is a 17.5 kDa, 157 amino acid member of the TNF superfamily of cytokines that is a potent lymphoid factor with effects on a wide range of target cells[1-3]. Active TNF- α is produced as both soluble and membrane-anchored trimers by macrophages, NK cells, and T- and B-lymphocytes. TNF exerts proinflammatory signals via binding and inducing trimerization of TNF-receptor 1 (TNF-R1) expressed on most normal and transformed cells, or to TNF-receptor 2 (TNF-R2), expressed on endothelial and most immune cells[2-3]. TNF signaling regulates hematopeiesis, differentiation, endothelial cell activation, apoptosis, lipid metabolism, tumor progression, and immune surveillance, and dysregulation of TNF or its receptors is implicated in numerous disease states including cancer, osteoporosis, autoimmune diseases, diabetes, and atherosclerosis[2-3].

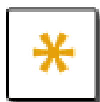
Principle

1. Samples and standards are added to wells coated with a monoclonal antibody specific for rat TNF-α. The plate is then incubated.
2. The plate is washed, leaving only bound rat TNF-α on the plate. A solution of biotinylated polyclonal antibody to rat TNF-α is then added. This binds the rat TNF-α captured on the plate. The plate is then incubated.
3. The plate is washed to remove excess antibody. A solution of HRP conjugate is added to each well, binding to the rat TNF-α polyclonal. The plate is again incubated.
4. The plate is washed to remove excess HRP conjugate. TMB Substrate solution is added. The substrate generates a blue color when catalyzed by the HRP.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is directly proportional to the level of rat TNF-α in the sample.

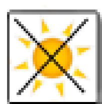
Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.



The standard should be handled with care due to the known and unknown effects of the antigen.



Activity of conjugate is affected by nucleophiles such as azide, cyanide, and hydroxylamine.



Protect substrate from prolonged exposure to light.

Stop solution is caustic. Keep tightly capped.

Materials Supplied

1. Assay Buffer 28

   27 mL, Catalog No. 80-1715

   Tris buffered saline containing detergents.

2. rat TNF-α Standard

   2,000 pg/vial, Catalog No. 80-1727

   Two vials containing 2,000 pg of recombinant rat TNF-α.

3. rat TNF-α Clear Microtiter Plate

   One Plate of 96 Wells, Catalog No. 80-1711

   A plate of break-apart strips coated with a mouse monoclonal antibody specific to rat TNF-α.

4. Wash Buffer Concentrate

   100 mL, Catalog No. 80-1287

   Tris buffered saline containing detergents.

5. rat TNF-α ELISA Antibody

   10 mL, Catalog No. 80-1712

   A solution of biotinylated polyclonal antibody to rat TNF-α.

6. rat TNF-α ELISA Conjugate

   10 mL, Catalog No. 80-1713

   A solution of streptavidin conjugated to horseradish peroxidase.

7. TMB Substrate

   10 mL, Catalog No. 80-0350

   A solution of 3,3’,5,5’ tetramethylbenzidine (TMB) and hydrogen peroxide.

8. Stop Solution 2

   10 mL, Catalog No. 80-0377

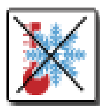
   A 1N solution of hydrochloric acid in water.

9. rat TNF-α Assay Layout Sheet

   1 each, Catalog No. 30-0245

10. Plate Sealer

    3 each, Catalog No. 30-0012



Reagents require separate storage conditions.

Storage

All components of this kit, **except the standard**, are stable at 4°C until the kit's expiration date. The standard **must** be stored at -20°C.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 5µl and 1,000µl.
3. Repeater pipet for dispensing 100µl.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Lint-free paper for blotting.
8. Microplate reader capable of reading at 450 nm.
9. Graph paper for plotting the standard curve.

Reagent Preparation

Bring all reagents to room temperature for at least 30 minutes prior to opening.

Plastic tubes must be used for standard preparation.

1. **Wash Buffer**

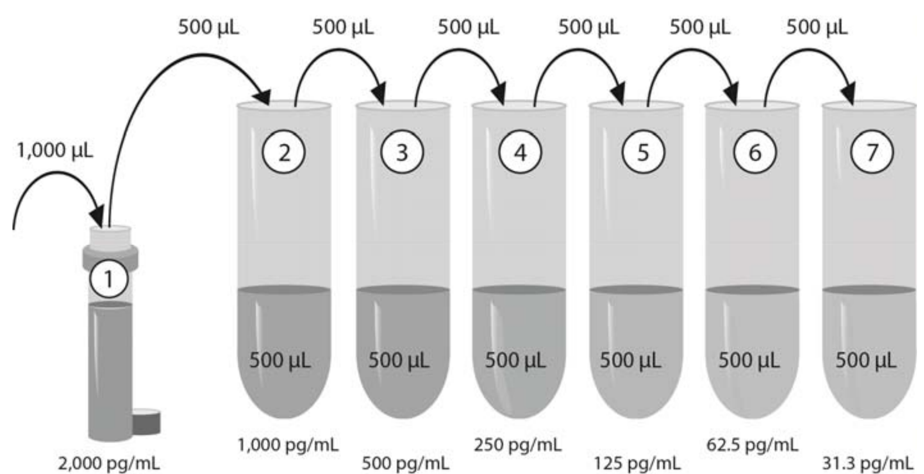
   Prepare the wash buffer by diluting 50 mL of the supplied Wash Buffer Concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. **rat TNF-α Standards**

   Two diluent options are available for the preparation of standards and samples. Please refer to the Sample Handling section on page 5 to determine which sample preparation is most suitable for your experiment. There will be some change in binding and sensitivity associated with running the standards and samples in media, and thus we do not recommend diluting samples any less than 1:8.

   Standard preparation for samples diluted 1:32 in Assay Buffer 28. Standards for supernates diluted 1:32 in Assay Buffer 28 are diluted directly into Assay Buffer 28. Add 1,000µl of assay buffer to the lyophilized rat TNF-α standard vial and vortex. Wait 5 minutes and vortex again prior to use. Label the vial Standard #1. Label six 12 x 75 mm polypropylene tubes #2 through #7. Pipet 500µl of Assay Buffer 28 into tubes #2 through #7. Add 500µl of reconstituted Standard #1 to tube #2 and vortex thoroughly. Add 500µl of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #7.

Standard preparation for samples diluted less than 1:32 in Assay Buffer 28. Standards may also be prepared in non-conditioned culture media equivalent to the samples, provided the same dilution of culture media into Assay Buffer 28 is used. Example: For samples diluted 1:8, prepare 5 mL of modified standard diluent by adding 625µl of non-conditioned media to 4.375 mL of Assay Buffer 28 in a 15 mL polypropylene tube. Vortex to mix, and retain 500µl for use as the S0 standard. Add 1,000µl of modified standard diluent to the lyophilized rat TNF-α standard vial and vortex. Wait 5 minutes and vortex again prior to use. Label the vial Standard #1. Label six 12 x 75 mm polypropylene tubes #2 through #7. Pipet 500µl of modified standard diluent into tubes #2 through #7. Add 500µl of reconstituted Standard #1 to tube #2 and vortex thoroughly. Add 500µl of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #7.



Diluted standards should be used within 30 minutes of preparation. The concentrations of rat TNF-α in each standard tube are labeled above.

Sample Handling

This kit is suitable for the detection of rat TNF-α in culture supernates with or without FBS. Samples containing greater than 10% FBS have not been tested. Supernatant samples require dilution of the media in Assay Buffer 28 before being run in the assay. Due to changes in binding and sensitivity associated with running standards and samples in media, we recommend diluting samples ≥1:8 in Assay Buffer 28.

Important note regarding standard curve preparation for samples diluted less than 1:32. Samples diluted less than 1:32 (e.g., 1:8 or 1:16) require dilution of the standards into a modified standard diluent, consisting of the same dilution of culture media in Assay Buffer 28 used for samples. See Reagent Preparation, page 4.

Expected % Recoveries for different media types spiked with rat TNF-α are shown below.

Sample	Diluent (Standard/Sample)	% Recovery	Recommended Dilution
RPMI (serum-free)	Assay Buffer 28/ Assay Buffer 28	86.2%	≥1:32
RPMI + 10% FBS	Assay Buffer 28/ Assay Buffer 28	90.5%	≥1:32
F-12K + 10% FBS	Modified Standard Diluent*/ Assay Buffer 28	113.5%	≥1:8

*See Reagent Preparation, page 4

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

Refer to the Assay Layout Sheet to determine the number of wells to be used.

Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C.

All standards and samples should be run in duplicate.

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

Pipet the reagents to the sides of the wells to avoid possible contamination.

1. Pipet 100µl of the appropriate sample diluent (Assay Buffer 28 or modified standard diluent) into the S0 (0 pg/mL standard) wells.
2. Pipet 100µl of Standards #1 through #7 to the bottom of the appropriate wells.
3. Pipet 100µl of the diluted samples to the bottom of the appropriate wells.
4. Seal the plate. Incubate for 1 hour on a plate shaker (~500 rpm) at room temperature.
5. Empty the contents of the wells and wash by adding 400µl of wash buffer to every well. Repeat 2 more times for a total of 3 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
6. Pipet 100µl of antibody into each well except the blank.
7. Seal the plate. Incubate for 1 hour on a plate shaker (~500 rpm) at room temperature.
8. Wash as above (Step 5).
9. Add 100µl of conjugate to each well except the blank.
10. Seal the plate. Incubate for 30 minutes on a plate shaker (~500 rpm) at room temperature.

Prior to the addition of the antibody, conjugate and substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

11. Wash as above (Step 5).
12. Pipet 100µl of substrate solution into each well.
13. Incubate for 30 minutes on a plate shaker (~500 rpm) at room temperature.
14. Pipet 100µl of stop solution into each well.
15. After blanking the plate reader against the substrate blank, read optical density at 450 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

Calculation of Results

Several options are available for the calculation of the concentrations of rat TNF-α in samples. We recommend that the data be handled by an immunoassay software package utilizing a 4-parameter logistic curve fitting program. If data reduction software is not readily available, the concentrations can be calculated as follows:

1. Calculate the average Net OD for each standard and sample by subtracting the average blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

2. Plot the average Net OD for each standard versus rat TNF-α concentration in each standard. Approximate a straight line through the points. The concentration of the unknowns can be determined by interpolation.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

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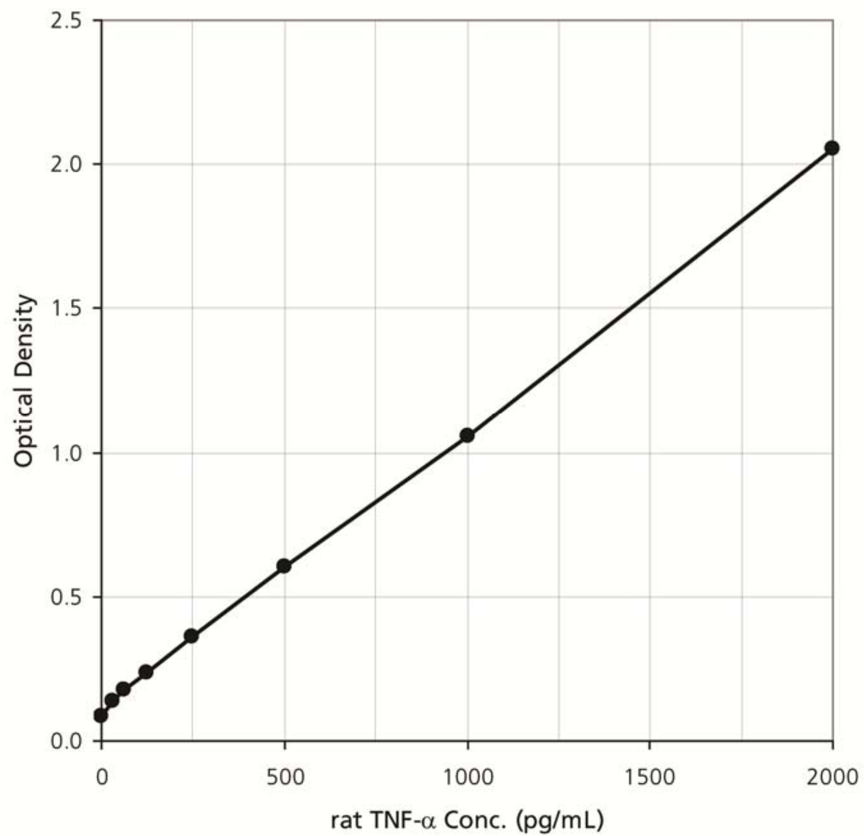
Typical Results in Assay Buffer 28

The results shown below are for illustration only and should not be used to calculate

results from another assay. The standard curve was prepared in Assay Buffer 28. Unknowns were Assay Buffer 28 spiked with rat TNF-α.

Sample	Net OD	rat TNF-α (pg/mL)
S0	0.88	**0**
S1	2.055	**2,000**
S2	1.058	**1,000**
S3	0.604	**500**
S4	0.360	**250**
S5	0.236	**125**
S6	0.174	**62.5**
S7	0.136	**31.3**
Unknown 1	1.074	**994**
Unknown 2	0.267	**165**

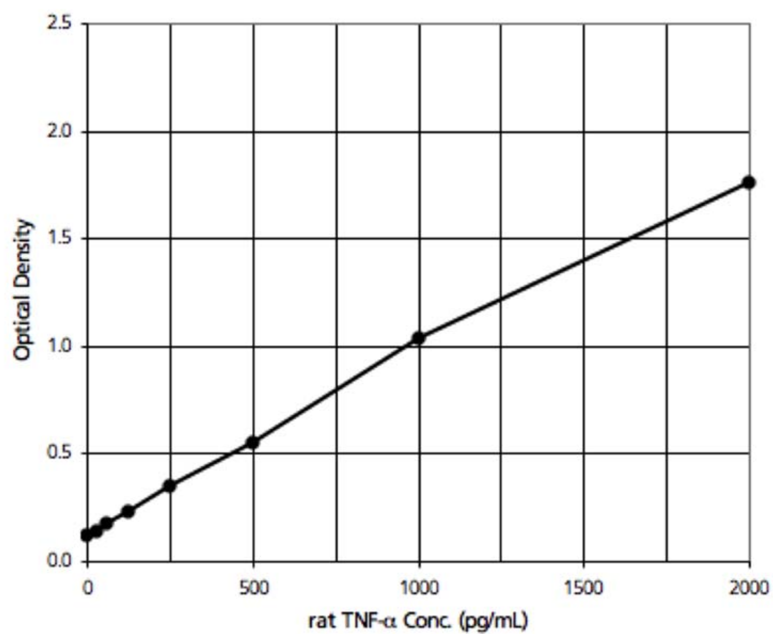


Typical Results in Diluted Culture Media

The results shown below are for illustration only and should not be used to calculate

results from another assay. The standard curve was prepared in modified standard diluent (non-conditioned 10% FBS media diluted 1:8 in Assay Buffer 28). Unknowns were non-conditioned 10% FBS culture media diluted 1:8 in Assay Buffer 28, spiked with rat TNF-α.

Sample	Net OD	rat TNF-α (pg/mL)
S0	0.118	**0**
S1	1.759	**2,000**
S2	1.034	**1,000**
S3	0.552	**500**
S4	0.346	**250**
S5	0.230	**125**
S6	0.175	**62.5**
S7	0.141	**31.3**
Unknown 1	0.751	**691**
Unknown 2	0.209	**110**



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Performance Characteristics

Specificity

The cross reactivities for a number of related compounds were determined by diluting cross reactants in the assay buffer at a concentration of 50,000 pg/mL. These samples were then measured in the assay. There is no significant cross reactivity with human TNF-α, human TNF-β, mouse TNF-α, or porcine TNF-α.

Sensitivity

Sensitivity was calculated as the ratio of the mean OD plus 2 standard deviations of twenty-four replicates of the 0 pg/mL standard to the mean of twenty-four replicates

of the lowest standard, multiplied by the concentration of that standard (31.3 pg/mL). This value was determined to be 12.0 pg/mL in Assay Buffer 28, and 26.7 pg/mL in non-conditioned 10% FBS culture media, diluted 1:8 into Assay Buffer 28.

Linearity

Assay Buffer 28 containing rat TNF-α at a concentration of 1500 pg/mL was serially diluted 1:2 in Assay Buffer 28 and measured using the assay. The results are shown in the table below.

Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
Neat	---------	1605.08	-----
1:2	802.54	814.03	101.4
1:4	401.27	414.28	103.2
1:8	200.64	217.51	108.4
1:16	100.32	111.48	111.1
1:32	50.16	48.69	97.1

Non-conditioned 10% FBS culture media was diluted 1:8 into Assay Buffer 28, then spiked with rat TNF-α to a concentration of 1500 pg/mL. The sample was then serially diluted 1:2 in the diluted culture media and measured in the assay. The results are shown in the table below.

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Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
Neat	---------	1473.49	-----
1:2	736.75	815.99	110.8
1:4	368.37	372.23	101.0
1:8	184.19	178.23	96.8
1:16	92.09	107.18	116.4
1:32	46.05	48.68	105.7

Precision

Intra-assay precision was determined in a single assay by evaluating 20 replicates of three spiked culture media samples (conditioned 10% FBS culture media, diluted 1:8 into Assay Buffer 28). %CVs obtained are listed below.

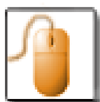
pg/mL	%CV
501.9	8.4
152.2	10.2
56.3	14.4

Inter-assay precision was determined in multiple assays over several days by evaluating three spiked culture media samples (conditioned 10% FBS culture media, diluted 1:8 into Assay Buffer 28).

pg/mL	%CV
781.3	5.8
236.6	5.5
104.3	12.3

References

1. Herbein, G. and O'Brien, W.A. (2000) Proc Soc Exp Biol Med. 223, 241-257.
2. Aggarwal, B.B. (2003) Nat Rev Immunol. 3, 745-756.
3. Taylor, P.C., Williams, R.O., Feldmann, M. (2004) Curr Opin Biotechnol. 15, 557-563.



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