# DRG® Rat TNFα (EIA-2358 / EIA-3168)

**Revised 10 Dec. 2010 rm (Vers. 4.1)** **For Research Use Only**

**This kit is intended for Research Use Only.**

**Not for use in diagnostic procedures.**

## Intended Use

The Rat Tumor Necrosis Factor-Alpha (Rt TNF-α) ELISA is to be used for determination of Rt TNF-α in rat serum, plasma, buffered solution, or cell culture medium. The assay will recognize both natural and recombinant Rt TNF-α.

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## Principle of the Method

The Rt TNF-α kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for Rt TNF-α has been coated onto the wells of the microtiter strips provided. Samples, including standards of known Rt TNF-α content, control specimens, and unknowns, are pipetted into these wells.
During the first incubation, the Rt TNF-α antigen binds to the immobilized (capture) antibody on one site. After washing, a biotinylated monoclonal antibody specific for Rt TNF-α is added. During the second incubation, this antibody binds to the immobilized Rt TNF-α captured during the first incubation.
After removal of excess second antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color.
The intensity of this colored product is directly proportional to the concentration of Rt TNF-α present in the original specimen.

## REAGENTS PROVIDED

Note: Store all reagents at 2-8°C.

| REAGENT | 96 Wells Test Kit | 192 Wells Test Kit |
|---|---|---|
| *Rt TNF -α* ***Standard***, recombinant Rt TNF -α.<br>Refer to vial label for quantity and reconstitution volume. | 2 vials | 4 vials |
| ***Standard Diluent Buffer***.<br>Contains 15 mM sodium azide; 25 mL per bottle. | 1 bottle | 2 bottles |
| ***Incubation Buffer***.<br>Contains 8 mM sodium azide; 12 mL per bottle. | 1 bottle | 1 bottle |
| *Rt TNF -α* ***High and Low Control***, recombinant Rt TNF-α lyophilized.<br>Refer to vial label for reconstitution volume and range.. | 2 vials | 2 vials |
| *Rt TNF -α* ***Antibody-Coated Wells***, 96 wells per plate. | 1 plate | 2 plates |
| *Rt TNF -α* ***Biotin Conjugate***, (Biotin.labeled anti-Rt TNF -α),<br>Contains 15 mM sodium azide; 11 ml per bottle. | 1 bottle | 2 bottles |
| ***Streptavidin-Peroxidase*** (HRP), (100x) concentrate.<br>Contains 3.3 mM thymol; 0.125 mL per vial | 1 vial | 2 vials |
| ***Streptavidin-Peroxidase*** **(HRP) Diluent**.<br>Contains 3.3 mM thymol; 25mL per bottle | 1 bottle | 1 bottle |
| ***Wash Buffer*** *concentrate (*25 x ); 100 ml per bottle | 1 bottle | 1 bottle |
| ***Stabilized Chromogen***. (Substrate Solution)<br>Tetramethylbenzidine (TMB); 25 ml per bottle | 1 bottle | 1 bottle |
| ***Stop Solution***; 25 ml per bottle | 1 bottle | 1 bottle |
| ***Plate Covers***, adhesive strips | 3 | 6 |

**Disposal Note:**

This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

## SUPPLIES REQUIRED BUT NOT PROVIDED

1. Microtiter plate reader capable of measurement at or near 450 nm.
2. Calibrated adjustable precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable for large assays).
3. Deionized or distilled water.
4. Plate washer; automated or manual (quirt bottle, manifold dispenser, etc.).
5. Data analysis and graphing software. Graph paper; linear (Cartesian), log-log, or semi-log, as desired.
6. Glass or plastic tubes for diluting and aliquoting standard.
7. Absorbent paper towels.
8. Calibrated beakers and graduated cylinders in various sizes.

## PROCEDURAL NOTES/LAB QUALITY CONTROL

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. **Microtiter plates should be allowed to come to room temperature before opening the foil bags.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2-8°C to maintain plate integrity.
3. Samples should be collected in pyrogen/endotoxin-free tubes.
4. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
5. When possible, avoid use of badly hemolized or lipemic sera. if large amounts of particulate matter are present, centrifuge or filter prior to analysis.
6. It is recommended that all standards, controls and samples be run in duplicate.
7. Serum/plasma samples that are >750 pg/ml should be diluted with *Incubation Buffer*.
8. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
9. Cover or cap all reagents when not in use.
10. **Do not mix or interchange different reagent lots from various kit lots.**
11. Do not use reagents after the kit expiration date.
12. Read absorbances within 30 minutes of assay completion.
13. The provided controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
14. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. **Never** insert absorbent paper directly into the wells.
15. Because *Stabilized Chromogen* is light sensitive, avoid prolonged exposure to light. Also avoid contact between Stabilized Chromogen and metal, or color may develop.

## SAFETY

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing infectious agents.

## DIRECTIONS FOR WASHING

**Incomplete washing will adversely affect the test outcome**. All washing must be performed with Wash Buffer provided.
Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into the bottom of each well. Take care not to scratch the inside of the well.
After aspiration, fill the wells with at least 0.4 mL of diluted wash solution. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under ASSAY METHOD. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.
Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.
If using an automated washer, the operating instructions for washing equipment should be carefully followed.

## REAGENT PREPARATION AND STORAGE

### A. Reconstitution and Dilution of Rt TNF-α Standard

**Note**: Either glass or plastic tubes may be used for standard dilutions.

1. Reconstitute standard to 2,000 pg/ml with *Standard Diluent Buffer*. Refer to standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Use standard within 1 hour of reconstitution.
2. Add 0.225 ml of the reconstituted standard to a tube containing 0.375 ml *Standard Diluent Buffer*. Label as 750 pg/ml Rt TNF-α. Mix.
3. Add 0.300 ml of *Standard Diluent Buffer* to each of 6 tubes labeled 375, 187.5, 93.8, 46.9, 23.4 and 11.7 pg/ml Rt TNF-α.
4. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

**Dilution of Rt TNF -α Standard**

| Standard | Add: | Into: |
|---|---|---|
| 750 pg/mL | Prepare as described in Step 2. | |
| 375pg/mL | 0.300 mLof the 750 pg/mL std. | 0.300 mLof the Diluent Buffer |
| 187.5 pg/mL | 0.300 mLof the 375 pg/mL std. | 0.300 mLof the Diluent Buffer |
| 93.8 pg/mL | 0.300 mLof the 187.5 pg/mL std. | 0.300 mLof the Diluent Buffer |
| 46.9 pg/mL | 0.300 mLof the 93.8 pg/mL std. | 0.300 mLof the Diluent Buffer |
| 23.4 pg/mL | 0.300 mLof the 46.9 pg/mL std. | 0.300 mLof the Diluent Buffer |
| 11.7 pg/mL | 0.300 mLof the 23.4 pg/mL std. | 0.300 mLof the Diluent Buffer |
| 0 pg/mL | 0.300 mLof the Diluent Buffer | An empty tube |

Discard all remaining reconstituted and diluted standards after completing assay. Return the Standard Diluent Buffer to the refrigerator.

**B. Sample Preparation and Treatment**

Serum/pasma samples:
In a microfuge tube, make a 1:2 dilution in *Incubation Buffer* and mix thoroughly prior to loading the samples in the microtiter wells.

Tissue culture supernatants:
In a microfuge tube, make a 1:2 dilution in *Standard Diluent Buffer* and mix thoroughly prior to loading the samples in the microtiter wells.

**C. Storage and Final Dilution of Streptavidin-HRP**

**Please Note:** The *Streptavidin-HRP* 100x concentrate is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow *Streptavidin-HRP* concentrate to reach room temperature. Gently mix. Pipette Streptavidin-HRP concentrate slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Dilute 10 µL of this 100x concentrated solution with 1 mL of Streptavidin-HRP Diluent for each 8-well strip used in the assay. Label as Streptavidin-HRP Working Solution.

For Example:

| # of 8-Well Strips | Volume of Streptavidin-HRP Concentrate | Volume of Diluent |
|---|---|---|
| 2 | 20 µL solution | 2 mL |
| 4 | 40 µL solution | 4 mL |
| 6 | 60 µL solution | 6 mL |
| 8 | 80 µL solution | 8 mL |
| 10 | 100 µL solution | 10 mL |
| 12 | 120 µL solution | 12 mL |

2. Return the unused Streptavidin-HRP concentrate to the refrigerator.

**D. Dilution of Wash Buffer**

Allow the 25x concentrate to reach room temperature and mix to unsure that any precipitated salts have re-dissolved. Dilute 1 volume of the 25 x wash buffer concentrate with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 liters, 100 mL may be diluted up to 2.5 liters). Label as Working Wash Buffer. Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

**ASSAY METHOD: PROCEDURE AND CALCULATIONS**:

**Be sure to read the Procedural Notes/Lab Quality Control section before carrying out the assay.**
Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.
**Note:** A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Add 100 µL of the Standard Diluent Buffer to zero wells. Well(s) reserved for chromogen blank should be left empty.
3. Add 100 µL of standards or controls to the appropriate microtiterwells.
4. Prior to loading samples, see "*Sample Preparation and Treatment"*.
5. Add 100 µl of prepared samples and/or controls to the appropriate microtiter wells.
6. Cover plate with *plate cover* and incubate for **2 hours at room temperature**.
7. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See DIRECTION FOR WASHING.
8. Pipette 100 µl of biotinylated anti-TNF-α (*Biotin Conjugate*) solution into each well except the chromogen blank(s).
9. Cover plate with *plate cover* and incubate for **1 hour at room temperature**.
10. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See DIRECTION FOR WASHING.
11. Add 100 µl Streptavidin-HRP Working Solution to each well except the chromogen blank(s). (Prepare the working dilution as described in REAGENT PREPARATION AND STORAGE, Section C).
12. Cover plate with the *plate cover* and incubate for **30 minutes at room temperature**.
13. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See DIRECTION FOR WASHING.
14. Add 100 µI of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.
15. Incubate for **30 minutes at room temperature and in the dark.**
    ***Please note:*** **Do not cover the plate with aluminium foil or metalized mylar**. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. value should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limited of the instrument. The O.D. values at 450 nm can only be read after the Stop Solution has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.
16. Add 100 µI of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.

17. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µl each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 30 minutes after adding the *Stop Solution*.
18. Plot on graph paper the absorbance of the standards against the standard concentration. (Optimally, the background absorbance may be subtracted from all data points, including standards, unknowns and controls, prior to plotting). Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provided the best curve fit.
19. Read the Rt TNF-α concentrations for unknown samples and controls from the standard curve plotted in Step 18. **Multiply value(s) obtained for sample(s) by 2 to correct for the 1:2 dilution in step 4.** (Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, multiplying the concentration found by the appropriate dilution factor.)

## TYPICAL DATA

The following data were obtained for the various standards over the range of 0 to 750 pg/mL Rt TNF-α.

| Standard Rt TNF -α (pg/mL) | Optical Density (450 nm) |
|---|---|
| 0 | 0.048 |
| | 0.054 |
| 11.7 | 0.116 |
| | 0.139 |
| 23.4 | 0.173 |
| | 0.193 |
| 46.9 | 0.297 |
| | 0.336 |
| 93.8 | 0.607 |
| | 0.579 |
| 187.5 | 1.010 |
| | 1.052 |
| 375 | 1.890 |
| | 1.966 |
| 750 | 3.177 |
| | 3.412 |

## LIMITATIONS OF THE PROCEDURE

Do note extrapolate the standard curve beyond the 1000 pg/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute serum/plasma samples >750 pg/mL with *Incubation Buffer* and tissue culture supernatant samples with *Standard Diluent Buffer*, re-analyze these and multiply results by the appropriate dilution factor.

The influence of various drugs, aberrant sera (hemolyzed, hyperlipimic, jaundiced, etc.) and the use of biological fluids in place of serum samples have not been thoroughly investigated. The rate of degradation of native Rt TNF-α in various matrices has not been investigated. The immunoassay literature contains frequent references to aberrant signals seen with

some sera, attributed to heterophilic antibodies. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.

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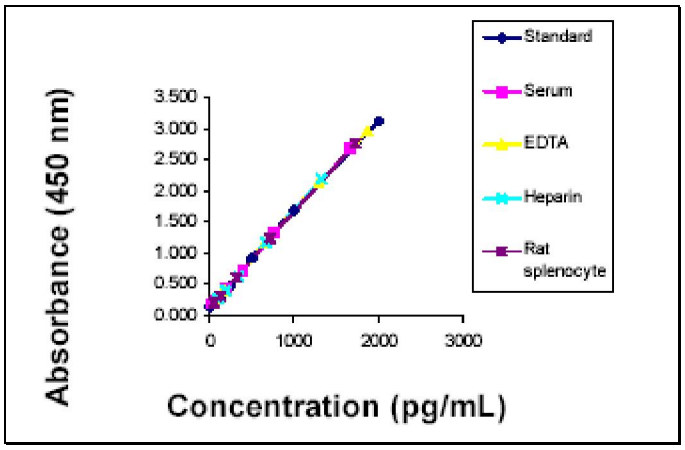
The recovery of natural Rt TNF-α added to tissue culture medium containing 1% fetal bovine serum averaged 104.8% (range: 97.1% to 112.7%, while the recovery of natural Rt TNF-α added to tissue culture medium containing 10% fetal bovine serum averaged 108.6% (range: 107.8% to 109.5%).

**PARALLELISM**

Supernatants from stimulated rat splenocytes were serially diluted in *Standard Diluent Buffer,* while rat serum and plasma samples spiked with natural Rt TNF-α were serially diluted in *Incubation Buffer*.
The optical density of each dilution was plotted against the Rt TNF-α standard curve. Parallelism was demonstrated by the figure below and indicated that the standard accurately reflects the Rt TNF-α content in natural samples.

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