DRG® Rat TNFα (EIA-3167, EIA-3158, EIA-4612)

Revised 7 May 2007 (vers. 4.0) **For Veterinary Use Only**

INTENDED USE

The DRG Mouse Tumor Necrosis Factor-α (Ms TNF-α) ELISA is to be used for the in vitro quantitative determination of Ms TNF-α in mouse serum, plasma, buffered solution, or cell culture medium. The assay will recognize both natural and recombinant Ms TNF-α.

INTRODUCTION

Mouse TNF-α, also known as cachectin, is a glycoprotein composed of 156 amino acids produced mainly by monocytes and activated macrophages. TNF-α has been shown to possess tumoricidal activity as well as a variety of physiologic effects with most major organ systems. In the central nervous system, TNF-α is involved in fever, anorexia, and alterations in pituitary hormone release. In the cardiovascular system, TNF-α plays a role in shock, acute respiratory distress, and capillary leakage syndrome (procoagulation). TNF-α is instrumental in the process of acute tubular necrosis and nephritis in the kidney and ischemia, colitis, and hepatic necrosis in the gastrointestinal system. Major TNF-α effects on metabolism include LPL suppression, protein catabolism, lipid catabolism, ACTH release, and insulin resistance. TNF-α is defined by its participation in inflammatory activities, cell toxicity, increased NK function, and mediation of TNF-α tumor toxicity. The ability to precisely measure TNF-α would provide information important to diagnose graft rejection and graft versus host disease. Accurate measurement of the changes in TNF-α levels of expression could aid in monitoring the pathogenesis of major infections and septic shock (1-7).

This kit has been configured for research use only and is not to be used in diagnostic procedures.
Read entire protocol before use.

PRINCIPLE OF THE METHOD

The DRG Ms TNF-α kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for Ms TNF-α has been coated onto the wells of the microtiter strips provided. Samples, including standards of known Ms TNF-α content, control specimens, and unknowns, are pipetted into these wells, followed by the addition of a biotinylated polyclonal second antibody.
During the first incubation, the Ms TNF-α antigen binds simultaneously to the immobilized (capture) antibody on one site, and to the solution phase biotinylated antibody on a second site.
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 Ms TNF-α present in the original specimen.

REAGENTS PROVIDED

Note: Store all reagents at 2 - 8°C.

Reagent	96 Test Kit	192 Test Kit	480 Test Kit
Ms TNF-α **Standard**, recombinant Ms TNF-α. Refer to vial label for quantity and reconstitution volume.	2 vials	4 vials	10 vials
Standard Diluent Buffer. Contains 15 mM sodium azide; 25 mL per bottle.	1 bottle	2 bottles	5 bottles
Ms TNF-α **High and Low Controls**, recombinant Ms TNF-α, lyophilized. Refer to vial label for reconstitution volume and range.	2 vials	2 vials	4 vials
Ms TNF-α **Antibody-Coated Wells**, 96 wells per plate.	1 plate	2 plates	5 plates
Ms TNF-α **Biotin Conjugate**, (Biotin-labeled anti-TNF-α). Contains 15 mM sodium azide; 6 mL per bottle.	1 bottle	2 bottles	5 bottles
Streptavidin-Peroxidase (HRP), (100x) concentrate. Contains 3.3 mM thymol; 0.125 mL per vial.	1 vial	2 vials	5 vials
Streptavidin-Peroxidase (HRP) Diluent. Contains 3.3 mM thymol; 25 mL per bottle.	1 bottle	1 bottle	3 bottles
Wash Buffer Concentrate (25x); 100 mL per bottle.	1 bottle	1 bottle	2 bottles
Stabilized Chromogen, Tetramethylbenzidine (TMB); 25 mL per bottle.	1 bottle	1 bottle	3 bottles
Stop Solution; 25 mL per bottle.	1 bottle	1 bottle	3 bottles
Plate Covers, adhesive strips.	3	6	15

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. Distilled or deionized water.
4. Plate washer: automated or manual (squirt 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 hemolyzed or lipemic serum and plasma samples. 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. Sample(s) that measure >1000 pg/ml should be diluted with Standard Diluent 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 controls provided 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 of 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 Ms TNF-α Standard

The DRG Ms TNF-α Standard is prepared from a highly purified E. coli-expressed recombinant protein. One microgram of the recombinant Ms TNF-α equals 624,000 arbitrary units of WHO reference preparation 88/532 (NIBSC, Hertfordshire, UK, EN6 3QG).

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

1. Reconstitute standard to 5000 pg/mL with Standard Diluent Buffer. Refer to the standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution.
   Use the standard within 1 hour of reconstitution.
2. Add 0.20 mL of the reconstituted standard to a tube containing 0.80 mL Standard Diluent Buffer.
   Label as 1000 pg/mL Ms TNF-α. Mix.
3. Add 0.300 mL of Standard Diluent Buffer to each of 6 tubes labelled 500, 250, 125, 62.5, 31.2 and 15.6 pg/mL Ms TNF-α.
4. Make serial dilutions of the standard as described in the following dilution table.
   Mix thoroughly between steps.

B. Dilution of Ms TNF-α Standard

Standard:	Add:	Into:
1000 pg/mL	Prepare as described in Step 2.	
500 pg/mL	0.300 mL of the 1000 pg/mL std.	0.300 mL of the Diluent Buffer
250 pg/mL	0.300 mL of the 500 pg/mL std.	0.300 mL of the Diluent Buffer
125 pg/mL	0.300 mL of the 250 pg/mL std.	0.300 mL of the Diluent Buffer
62.5 pg/mL	0.300 mL of the 125 pg/mL std.	0.300 mL of the Diluent Buffer
31.2 pg/mL	0.300 mL of the 62.5 pg/mL std.	0.300 mL of the Diluent Buffer
15.6 pg/mL	0.300 mL of the 31,2 pg/mL std.	0.300 mL of the Diluent Buffer
0 pg/mL	0.300 mL of the Diluent Buffer	An empty tube

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

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. Within 1 hour of use, 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 ensure that any precipitated salts have redissolved. Dilute 1 volume of the 25x 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. For the standard curve, add 100 µL of standards to the appropriate microtiter wells. Add 100 µL of the Standard Diluent Buffer to zero wells. Well(s) reserved for chromogen blank should be left empty.
3. Serum, plasma and cell and tissue culture samples require a 2-fold dilution in Standard Diluent Buffer. For these samples add 50 µL of Standard Diluent Buffer to each well, followed by 50 µL of sample. Tap on the side of the plate to mix.
4. Buffered solutions and controls may be assayed neat; add 100 µL of each buffered solution and each control to the appropriate microtiter wells.
5. Pipette 50 µL of biotinylated anti-TNF-α (Biotin Conjugate) solution into each well except the chromogen blank(s). Tap on the side of the plate to mix.
6. Cover plate with plate cover and incubate for **90 minutes at room temperature**.

7. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See DIRECTIONS FOR WASHING.
8. 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.)
9. Cover plate with the plate cover and incubate for **30 minutes at room temperature**.
10. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See DIRECTIONS FOR WASHING.
11. Add 100 µL of Stabilized Chromogen to each well. The liquid in the wells will begin to turn blue.
12. Incubate for **30 minutes at room temperature and in the dark**.
    Please Note: **Do not cover the plate with aluminum 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. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits 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.
13. Add 100 µL of Stop Solution to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
14. 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.
15. 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 provides the best curve fit.
16. Read the Ms TNF-α concentrations for unknown samples and controls from the standard curve plotted in step 15. **Multiply value(s) obtained for serum, plasma, or tissue culture sample(s) by 2 to correct for the 1:2 dilution in steps 3.** (Samples producing signals greater than that of the highest standard (1000 pg/mL) should be further diluted in Standard Diluent Buffer and reanalyzed. Multiply 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 1000 pg/mL Ms TNF-α.

Standard Ms TNF-α (pg/mL)	Optical Density (450 nm)
0	0.088 0.086
15.6	0.249 0.272
31.2	0.381 0.429
62.5	0.591 0.643
125	1.051 1.078
250	1.722 1.857
500	2.522 2.764
1000	3.249 3.516

LIMITATIONS OF THE PROCEDURE

Do not 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 samples with concentrations exceeding the linear portion of the Standard Curve with Standard Diluent Buffer; reanalyze these and multiply results by the appropriate dilution factor.
The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum samples have not been thoroughly 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.
This kit is for research use only.
Not for human therapeutic or diagnostic use.

PERFORMANCE CHARACTERISTICS

SENSITIVITY

The minimum detectable dose of Ms TNF-α is < 3 pg/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times.

PRECISION

1. Intra-Assay Precision

Samples of known Ms TNF-α concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	50.0	149.5	629.2
SD	2.9	9.5	51.1
%CV	5.9	6.4	8.1

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	53.8	152.0	650.6
SD	4.7	9.5	57.3
%CV	8.7	6.3	8.8

LINEARITY OF DILUTION

Mouse serum, citrate plasma, heparinized plasma, and tissue culture medium spiked with natural Ms TNF-α were serially diluted in Standard Diluent Buffer over the range of the assay.
Linear regression analysis of samples versus the expected concentration yielded average correlation coefficients of 0.999 for serum, heparinized plasma and tissue culture medium, and 0.993 for citrate plasma.

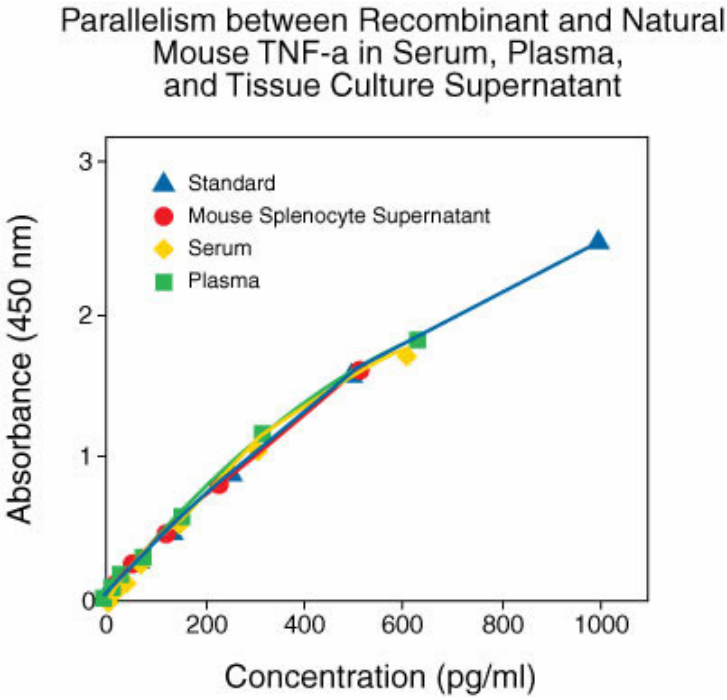
RECOVERY

The recoveries of Ms TNF-α added to mouse serum, citrate plasma, heparinized plasma, and tissue culture media containing 10% fetal bovine serum were measured with the Ms TNF-α ELISA.

Sample Type	Range	Average Recovery
Serum	84.9-99.4	92.8%
Citrate plasma	74.8-104.5	88.3%
Heparinized plasma	74.2-118	91.3%
RPMI + 10% fetal bovine serum	104.1-	109%

PARALLELISM

Supernatants from stimulated mouse splenocytes as well as mouse serum and plasma spiked with natural Ms TNF-α were serially diluted in Standard Diluent Buffer. The optical density of each dilution was plotted against the Ms TNF-α standard curve. Parallelism was demonstrated and indicated that the standard accurately reflects the Ms TNF-α content in natural samples.


Parallelism between Recombinant and Natural
Mouse TNF-a in Serum, Plasma,
and Tissue Culture Supernatant
Standard
Mouse Splenocyte Supernatant
Serum
Plasma
Absorbance (450 nm)
0
1
2
3
0
200
400
600
800
1000
Concentration (pg/ml)

SPECIFICITY

Buffered solutions of a panel of substances ranging in concentrations from 0.50 to 30 ng/mL were assayed with the Ms TNF-α ELISA kit and found to have no cross-reactivity:
Mouse EGF, FGF basic, G-CSF, GM-CSF, KC, IFN-γ , IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IP-10, MCP-1, MIG, MIP-1α, MIP-1β, MIP-3β, PDGF-BB, RANTES and VEGF;
Human TNF-α; and
Swine TNF-α.
Recombinant rat TNF-α protein demonstrated 30% cross-reactivity with this kit.

HIGH DOSE HOOK EFFECT

No hook effect was observed with concentrations up to 250 ng/mL.

EXPECTED VALUES

Twenty mouse serum and plasma samples were evaluated for detectable levels of Ms TNF-α in this assay.
The mean value of the serum and plasma samples measured less than the lowest Ms TNF-α standard, 15.6 pg/mL.
Supernatants from mouse splenocytes cultured in the presence of 50 ng/mL PMA and 250 ng/mL calcium ionophore for 72 hours were assayed for Ms TNF-α and measured an average of 1142 pg/mL.

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Important Information
Unless otherwise indicated, these products are for research use only and are not intended for human or animal diagnostic, therapeutic or commercial use.

Symbols used with DRG Assays

Symbol	English	Deutsch	Français	Español	Italiano
	Consult instructions for use	Gebrauchsanweisung beachten	Consulter les instructions d'utilisation	Consulte las instrucciones de uso	Consultare le istruzioni per l'uso
	European Conformity	CE-Konfirmitäts-kennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
	In vitro diagnostic device	In-vitro-Diagnostikum	Usage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
	For research use only	Nur für Forschungszwecke	Seulement dans le cadre de recherches	Sólo para uso en investigación	Solo a scopo di ricerca
	Catalogue number	Katalog-Nr.	Numéro de catalogue	Número de catálogo	Numero di Catalogo
	Lot. No. / Batch code	Chargen-Nr.	Numéro de lot	Número de lote	Numero di lotto
	Contains sufficient for <n> tests/	Ausreichend für "n" Ansätze	Contenu suffisant pour "n" tests	Contenido suficiente para <n> ensayos	Contenuto sufficiente per "n" saggi
	Storage Temperature	Lagerungstemperatur	Température de conservation	Temperatura de conservación	Temperatura di conservazione
	Expiration Date	Mindesthaltbarkeits-datum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Distributed by	Distributor	Vertreiber	Distributeur	Distribuidor	Distributore
Content	Content	Inhalt	Conditionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità

Symbol	Portugues	Dansk	Svenska	Ελληνικά
	Consulte as instruções de utilização	Se brugsanvisning	Se bruksanvisningen	Εγχειρίδιο χρήστη
	Conformidade com as normas europeias	Europaeisk overensstemmelse	Europeisk överensstämmelse	Ευρωπαϊκή Συμμόρφωση
	Catálogo n.º	Katalognummer	Katalog nummer	Αριθμός καταλόγου
	No do lote	Lot nummer	Batch-nummer	Αριθμός Παρτίδος
		Indeholder tilsttrækkeligt til "n" test	Innehåller tillräckligt till "n" tester	Περιεχόμενο επαρκές για «n» εξετάσεις
	Temperatura de conservação	Opbevarings-temperatur	Förvaringstempratur	Θερμοκρασία αποθήκευσης
	Prazo de validade	Udløbsdato	Bäst före datum	Ημερομηνία λήξης
	Fabricante	Producent	Tillverkare	Κατασκευαστής
Distributed by				
Content	Conteúdo	Indhold	Innehåll	Περιεχόμενο
Volume/No.	Volume/Número	Volumen/antal	Volym/antal	Όγκος/αριθ..