DRG® TRAIL human ELISA (EIA-4009)

Revised 30 Jan. 2006 **RUO**

INTRODUCTION

TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is a recently identified member of the TNF gene superfamily. Five different receptors have been identified for TRAIL. Two receptors, DR4 and DR5, are transmembrane proteins containing death domain similar to FAS and other TNF family receptors. Two other receptors, DcR1 and DcR2, act like decoy proteins for TRAIL binding because they lack the death domain. TRAIL can also bind, though weakly, to osteoprotegrin (OPG), a soluble receptor, which plays a role in osteoclastogenesis.

TRAIL induces apoptosis in various tumor cell lines, whereas most primary cells seem to be resistant. TRAIL-mediated apoptosis occurs following its binding to DR4 or DR5 receptors. The mechanism of apoptosis involves activation of caspase-8 and subsequent activation of effector caspases. Also, NF-κB and JNK activation play a role in the TRAIL signaling pathway.

TRAIL expression is detectable in many normal organs and tissues. Several studies suggest that TRAIL may play a physiological role by contributing to immune privilege, normal cellular development, and inhibition of autoimmune responses. The expression of TRAIL is upregulated in activated T cells, B cells, NK cells, monocytes and macrophages. LPS activation stimulates the release of soluble TRAIL from monocytes and macrophages, suggesting a role of TRAIL in their cytotoxic/phagocytic function. Also, TRAIL expression is increased in transformed cell lines and various diseases including cancer and autoimmune disorders. TRAIL may also be involved in activation-induced T cell death during HIV infection. Certain anti-cancer agents also upregulate TRAIL and TRAIL receptor expression on tumor cells, thus sensitizing cells to apoptosis.

INTENDED USE

The DRG® Human TRAIL (hTRAIL) ELISA is to be used for the *in vitro* quantitative determination of hTRAIL in human serum, plasma, cell extracts, buffered solution, or cell culture medium.

The assay will recognize both natural and recombinant hTRAIL.

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® human TRAIL kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for human TRAIL has been coated onto the wells of the microtiter strips provided. Samples, including standards of known human TRAIL content, control specimens, and unknowns, are pipetted into these wells. During the first incubation, the hTRAIL antigen binds to the immobilized (capture) antibody on one site. After washing, a biotinylated polyclonal antibody specific for hTRAIL is added. During the second incubation, this antibody binds to the immobilized hTRAIL 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 third 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 hTRAIL present in the original specimen.

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REAGENTS PROVIDED

Note: *Store all reagents at 2 - 8°C.*

Reagent	***96 Test Kit***
hTRAIL Standard, purified recombinant hTRAIL expressed in *E. coli*. Refer to vial label for quantity and reconstitution volume.	2 vials
Standard Diluent Buffer. Contains 15 mM sodium azide; 25 mL per bottle.	1 bottle
hTRAIL Antibody-Coated Wells, 96 wells per plate	1 plate
hTRAIL Biotin Conjugate (Biotin-labeled anti-TRAIL). Contains 15 mM sodium azide; 11 mL per bottle.	1 bottle
Streptavidin-Peroxidase (SAV-HRP), (100x) concentrate. Contains 3.3 mM thymol; 0.125 mL per vial.	1 vial
Streptavidin-Peroxidase (HRP) Diluent. Contains 3.3 mM thymol; 25 mL per bottle.	1 bottle
Wash Buffer Concentrate (25x); 100 mL per bottle	1 bottle
Stabilized Chromogen, Tetramethylbenzidine(TMB); 25 mL per bottle.	1 bottle
Stop Solution; 25 mL per bottle	1 bottle
Plate Covers, adhesive strips.	3

SUPPLIES - 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 H2O.
4. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
5. 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 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. Samples that are >3000 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. Read absorbances within 2 hours of assay completion.
12. Do not use reagents after the kit expiration date.
13. In-house 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. If your automated washer allows, 30 second soak cycles should be programmed into the wash cycle.

PROCEDURE FOR EXTRACTION OF PROTEINS FROM CELLS

A. Recommended Formulation of Cell Extraction Buffer:

10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM Na4P2O7
2 mM Na3VO4
1% Triton X-100
10% glycerol
0.1% SDS
0.5% deoxycholate
1 mM PMSF (stock is 0.3 M in DMSO)

Protease inhibitor cocktail (ex., Sigma Cat. # P-2714) (reconstituted according to manufacturer's guideline). Add 250 µL per 5 mL Cell Extraction Buffer.
This buffer is stable for 2-3 weeks at 4oC or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at -20oC. When stored frozen, the Cell Extraction Buffer should be thawed on ice. Important: add the protease inhibitors just before using.
The stability of protease inhibitor supplemented Cell Extraction Buffer is 24 hours at 4oC. PMSF is very unstable and must be added prior to use, even if added previously.

B. Protocol for Cell Extraction
This protocol has been applied to several cell lines with the Cell Extraction Buffer above. Researchers may optimize the cell extraction procedures that work best in their hands.

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash cells twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at -80ºC and lysed at a later date.)
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10 minute intervals. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of TRAIL. Under these conditions, use of a 1:10 dilution of cell extract with Standard Diluent Buffer (See **Assay Method**) is sufficient for the detection of TRAIL. Other extraction methods can be employed, but the initial dilution to be made must be optimized.
5. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4°C.
6. Aliquot the clear lysate to clean microfuge tubes. These samples are ready for assay. Lysates can be stored at -80oC. Avoid multiple freeze/thaw cycles.

REAGENT PREPARATION AND STORAGE
A. Reconstitution and Dilution of human TRAIL Standard
Note: Either glass or plastic tubes may be used for standard dilutions.

1. Reconstitute standard to 3000 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 0.300 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 1500, 750, 375, 187.5, 93.7, and 46.8 pg/mL hTRAIL.
3. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution of human TRAIL Standard

Standard:	**Add:**	**Into:**
3000 pg/mL	Prepare as described in step 1.	
1500 pg/mL	0.300 mL of the 3000 pg/mL std.	0.300 mL of the Diluent Buffer
750 pg/mL	0.300 mL of the 1500 pg/mL std.	0.300 mL of the Diluent Buffer
375 pg/mL	0.300 mL of the 750 pg/mL std.	0.300 mL of the Diluent Buffer
187.5 pg/mL	0.300 mL of the 375 pg/mL std.	0.300 mL of the Diluent Buffer
93.7 pg/mL	0.300 mL of the 187.5 pg/mL std.	0.300 mL of the Diluent Buffer
46.8 pg/mL	0.300 mL of the 93.7 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 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. 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. If using cell extracts containing SDS or other ionic detergents, dilute samples at least 1:10 with *Standard Diluent Buffer* (e.g., add 20 µL of sample to 180 µL *Standard Diluent Buffer*). For plasma and serum samples, dilute 1:2 with *Standard Diluent Buffer*. Cell culture supernatants containing 5-10% serum should be tested neat.
3. Add 100 µL of the *Standard Diluent Buffer* to zero wells. Well(s) reserved for chromogen blank should be left empty.

4. Add 100 µL of standards or samples (serum, plasma, all extracts, buffered solutions and cell culture medium) to the appropriate microtiter well(s). Tap gently on side of plate to mix. (See **REAGENT PREPARATION AND STORAGE**, Section B.)
   Note: Individual samples may require a greater or lesser dilution to fall within the range of the assay.
5. Cover plate with *plate cover* and incubate for **2 hours at room temperature**.
6. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
7. Pipette 100 µL of biotinylated anti-TRAIL (*Biotin Conjugate*) solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.
8. Cover plate with *plate cover* and incubate for **1 hour at room temperature**.
9. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
10. 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.)
11. Cover plate with the *plate cover* and incubate for **30 minutes at room temperature**.
12. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
13. Add 100 µL of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.
14. 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.
15. 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.
16. 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 2 hours after adding the *Stop Solution*.
17. Plot on graph paper the absorbance of the standards against the standard concentration. (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.
18. Read the hTRAIL concentrations for unknown samples and controls from the standard curve plotted in step 17. **To correct for dilution, multiply value(s) obtained for sample(s) by dilution factor utilized in step 2.** (Samples producing signals greater than that of the highest standard (3000 pg/mL) should be further diluted in *Standard Diluent Buffer* and re-analyzed, multiplying the concentration found by the appropriate dilution factor.)

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TYPICAL DATA

The following data were obtained for the various standards over the range of 0 to 3000 pg/mL hTRAIL.

Standard hTRAIL (pg/mL)	Optical Density (450 nm)
0	0.177
46.8	0.243
93.7	0.302
187.5	0.408
375	0.615
750	0.952
1500	1.698
3000	2.944

LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 3000 pg/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples >3000 pg/mL with *Standard Diluent Buffer*; re-analyze 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 rate of degradation of native hTRAIL 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.

This kit is for research use only.
Not for human therapeutic or diagnostic use.

PERFORMANCE CHARACTERISTICS

SENSITIVITY

The minimum detectable dose of TRAIL is <20 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 TRAIL concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	282	1121	2230
SD	19.7	101.2	143.4
%CV	6.9	9.0	6.4

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision
Samples were assayed 48 times in multiple assays to determine precision between assays.

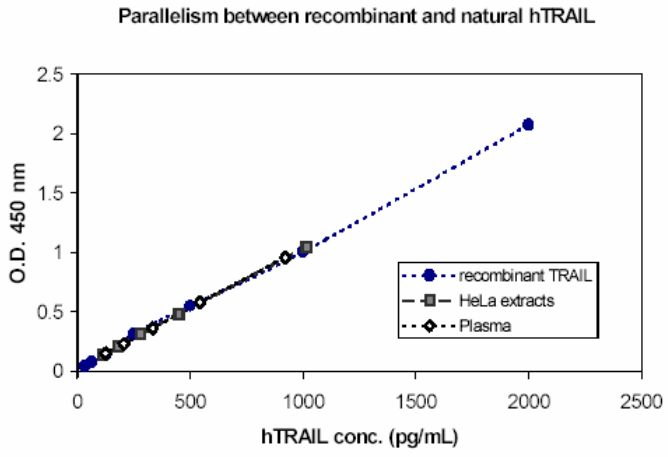
	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	275	1066	2293
SD	13.3	54.6	68.3
%CV	4.8	5.1	2.9

SD = Standard Deviation
CV = Coefficient of Variation

LINEARITY OF DILUTION
Human serum and tissue culture medium containing 10% fetal calf serum were spiked with hTRAIL and serially diluted in *Standard Diluent Buffer* over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99 in both cases.

RECOVERY
The recovery of hTRAIL added to human serum averaged 85%, while the recovery of hTRAIL added to tissue culture medium containing 10% fetal calf serum averaged 105%. The recovery of hTRAIL added to citrate or heparin plasma averaged 77.1 and 98.4%, respectively. EDTA plasma yielded poor recovery and is not recommended.

PARALLELISM
Natural hTRAIL from plasma or HeLa cell lysate were serially diluted in *Standard Diluent Buffer.* The optical density of each dilution was plotted against the standard curve. Parallelism between the natural and recombinant proteins was demonstrated by the figure below and indicated that the standard accurately reflects natural hTRAIL content in samples.



SPECIFICITY
Buffered solutions of a panel of substances at 80,000 pg/mL were assayed with the DRG® human TRAIL kit. The following substances were tested and found to have no cross-reactivity:
human DR4 (TRAIL receptor 1), DR5 (TRAIL receptor 2), basic FGF, IFN-γ, VEGF, TGF-α, TGF-β, FasL; mouse G-CSF, TNF-α; bovine FGF.

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EXPECTED VALUES

Seven sera and ten plasma (heparin) samples from healthy individuals were evaluated in this assay. The values for sera ranged from 100 to 1800 pg/mL. The values for plasma (heparin) ranged from 300 to 1000 pg/mL.
A limited number of commercially available pooled serum samples measured 100 to 500 pg/mL.
A limited number of cell culture supernatants ranged from 0 to 200 pg/mL.
Cell extracts of several human cell lines were also tested (diluted 1:10 in *Standard Diluent*). The values (after correction for dilution) ranged from 0.45 to 15 ng/mL at 1 mg/mL of total protein.

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