DRG® Swine Tumor Necrosis Factor-alpha (swTNF-α)
(EIA-3186 = 96 wells)
(EIA-3187 = 192 wells)

Revised 9 July 2011 rm (Vers. 5.1) **For Veterinary Use Only**

This kit is intended for Research Use Only.

Not for use in diagnostic procedures.

INTENDED USE

The DRG Porcine TNF-α (Sw TNF-α) ELISA is to be used for the in vitro quantitative determination of Sw TNF-α in swine serum, EDTA plasma, buffered solution, or cell culture medium.
The assay will recognize both natural and recombinant Sw TNF-α.

PRINCIPLE OF THE METHOD

The DRG Porcine TNF-α kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for Sw TNF-α has been coated onto the wells of the microtiter strips provided. Samples, including standards of known Sw TNF-α content, control specimens, and unknowns, are pipetted into these wells.
During the first incubation, the Sw TNF-α antigen binds to the immobilized (capture) antibody on one site. After washing, a biotinylated monoclonal antibody specific for Sw TNF-α is added. During the second incubation, this antibody binds to the immobilized Sw 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 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.

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REAGENTS PROVIDED

Note: Store all reagents at 2 - 8°C.

Reagent	96 Test Kit	192 Test Kit
Sw TNF-α **Standard**, recombinant E. coli Sw TNF-α lyophilized. Refer to vial label for quantity and reconstitution volume.	2 vials	4 vials
Standard Diluent Buffer. Contains 8 mM sodium azide; 25 mL per bottle.	1 bottle	2 bottles
Incubation Buffer. Contains 8 mM sodium azide; 11 mL per bottle.	1 bottle	1 bottle
Sw TNF-α High and Low **Controls**, recombinant E. coli Sw TNF-α lyophilized. Refer to vial label for reconstitution volume and range.	2 vials	4 vials
Sw TNF-**αAntibody-Coated Wells**, 96 wells per plate.	1 plate	2 plates
Sw TNF-α **Biotin Conjugate** (Biotin-labeled anti-Sw TNF-α) (**100x concentrate**). Contains 8 mM sodium azide; 0.125 mL per vial.	1 vial	2 vials
Sw TNF-α **Biotin Conjugate Diluent Buffer**. Contains 8 mM sodium azide; 12.5 mL per bottle.	1 bottle	2 bottles
Streptavidin-Peroxidase (HRP), (100x) concentrate. Contains 3.3 mM thymol; 0.125 mL per vial.	1 vial	2 vials
Streptavidin-Peroxidase (HRP) Diluent. Contains 3.3 mM thymol; 25 mL per bottle.	1 bottle	1 bottle
Wash Buffer Concentrate (25x); 100 mL per bottle.	1 bottle	1 bottle
Stabilized **Chromogen**, Tetramethylbenzidine (TMB); 25 mL per bottle.	1 bottle	1 bottle
Stop Solution; 25 mL per bottle.	1 bottle	1 bottle
Plate Covers, adhesive strips.	4	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.

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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 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 >1500 pg/mL should be diluted with Standard Diluent Buffer for serum/plasma samples and with corresponding medium for cell culture samples or buffered solutions.
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 2 hours 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 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

The Sw TNF-α standard was calibrated against the mass of a highly purified E. coli recombinant protein.

Reconstitution and Dilution of Sw TNF-α Standard

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

1. Reconstitute standard to 7,500 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 0.1 mL of the reconstituted standard to a tube containing 0.4 mL Standard Diluent Buffer.
   Label as 1500 pg/mL Sw TNF-α. Mix.
3. Add 0.250 mL of Standard Diluent Buffer to each of 6 tubes labeled 750, 375, 187.5, 93.8, 46.9 and 23.4 pg/mL Sw TNF-α.
4. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

Dilution of Sw TNF-α Standard

Standard:	Add:	Into:
1500 pg/mL	Prepare as described in Step 2.	
750 pg/mL	0.250 mL of the 1500 pg/mL std.	0.250 mL of the Diluent Buffer
375 pg/mL	0.250 mL of the 750 pg/mL std.	0.250 mL of the Diluent Buffer
187.5 pg/mL	0.250 mL of the 375 pg/mL std.	0.250 mL of the Diluent Buffer
93.8 pg/mL	0.250 mL of the 187.5 pg/mL std.	0.250 mL of the Diluent Buffer
46.9 pg/mL	0.250 mL of the 93.8 pg/mL std.	0.250 mL of the Diluent Buffer
23.4 pg/mL	0.250 mL of the 46.9 pg/mL std.	0.250 mL of the Diluent Buffer
0 pg/mL	0.250 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.

Reconstitution of Sw TNF-α controls

Reconstitute controls with 0.5 mL distilled or deionized water. Refer to controls vial labels for values and acceptable ranges in pg/mL.
Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution.
Once reconstituted, aliquot and store at -20°C or below. Avoid repeated freeze-thaw cycles.

Preparation of the Biotin Conjugate Working Solution

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

   For Example:

# of 8-Well Strips	Volume of Biotin Conjugate 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 Biotin Conjugate concentrate and the Biotin Conjugate Diluent Buffer to the refrigerator.

Storage and Final Dilution of Streptavidin-HRP

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

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.)

Note: Please add the reagents in the following order:

2. Add 50 µL of the *Incubation Buffer* to zero wells and to the wells corresponding to standards, controls, swine serum, plasma, buffered solution and cell culture samples.
   Well(s) reserved for chromogen blank should be left empty.
3. Add 100 µL of the *Standard Diluent Buffer* to zero wells.
   Well(s) reserved for chromogen blank should be left empty.
4. For the standards, add 100 µL of sample to the appropriate microtiter wells. (See REAGENT PREPARATION AND STORAGE, Section 2.)
   For controls, swine serum, plasma, cell culture medium or buffered solutions, add 50 µL sample to each well followed by 50 µL of *Standard Diluent Buffer*. Tap gently on the side of plate to mix.
5. Cover plate with plate cover and incubate for **3 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 Sw TNF-α Biotin Conjugate Working Solution into each well except the chromogen blank(s). (Prepare the working dilution as described in REAGENT PREPARATION AND STORAGE, Section 4.) 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 5.)
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. (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.
18. Read the Sw TNF-α concentrations for unknown samples and controls from the standard curve plotted in step 17. **Multiply value(s) obtained for controls and sample(s) by 2 to correct for the 1:2 dilution in step 4**. (Samples producing signals greater than that of the highest standard (1500 pg/mL) should be further diluted in Standard Diluent Buffer for serum/plasma samples or corresponding medium for cell culture samples or buffered solutions, and reanalyzed, 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 1500 pg/mL Sw TNF-α.
Standard Optical Density Sw TNF-α (pg/mL) (450 nm)

Standard Sw TNF-□ (pg/mL)	Optical Density (450 nm)
0	0.055 0.053
23.4	0.105 0.107
46.9	0.150 0.154
93.8	0.276 0.297
187.5	0.502 0.509
375.0	0.952 0.997
750.0	1.747 1.799
1500.0	3.502 3.568

LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 1500 pg/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain.
Dilute samples >1500 pg/mL with Standard Diluent Buffer for serum/plasma samples and with corresponding medium for cell culture samples or buffered solutions; reanalyze these and multiply results by the appropriate dilution factor.
The influence of various drugs, aberrant sera or plasma (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 Sw 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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PERFORMANCE CHARACTERISTICS

Sensitivity

The minimum detectable dose of Sw 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

Intra-Assay Precision

Samples of known Swine TNF-α concentration were assayed in replicates of 24 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	56	149	614
SD	3.2	9.2	37
%CV	5.7	6.2	6.0

SD = Standard Deviation CV = Coefficient of Variation

Inter-Assay Precision

Samples were assayed 16 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	63	168	695
SD	5.2	11	50
%CV	8.2	6.5	7.2

Linearity of Dilution

Swine serum or cell culture samples containing Sw TNF-α were serially diluted over the range of the assay in Standard Diluent Buffer or RPMI containing 10% fetal bovine serum, respectively.
Linear regression analysis of samples versus the expected concentration yielded an average correlation coefficient of 0.99.

	Serum				**Cell Culture**		
Dilution	**Measured (pg/mL)**	**Expected (pg/mL)**	**% Expected**	**Dilution**	**Measured (pg/mL)**	**Expected (pg/mL)**	**% Expected**
1/2	886	-	-	1/20	994	-	-
1/4	444	443	100	1/40	490	497	98.6
1/8	222	222	100	1/80	246	249	98.8
1/16	105	111	94.6	1/160	136	124	109
1/32	53	55	96.4	1/320	58	62	93.5

Recovery

The recovery of Sw TNF-α added to swine serum and EDTA plasma averaged 91% or 81%, respectively.
The recovery of Sw TNF-α added to tissue culture medium containing 1% fetal bovine serum averaged 102%, while the recovery of Sw TNF-α added to tissue culture medium containing 10% fetal bovine serum averaged 98%.

Specificity

Buffered solutions of a panel of substances at 50 ng/mL were assayed with the DRG Sw TNF-α kit. The following substances were tested and found to have no cross-reactivity:
Swine IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-15;
mouse TNF-α;
rat TNF-α;
human TNF-α.

Expected Values

Serum/Plasma
Each laboratory must establish its own normal values.
For guidance, the mean of 20 normal sera was 53 pg/mL (range: from 16 to 138 pg/mL).
The mean of 20 normal EDTA plasma samples was 65 pg/mL (range: from undetectable to 189 pg/mL).
Cell culture supernatants were evaluated in this assay.
Swine Whole Blood (WB) cells were cultured for 24, 48 or 72 hours in RPMI supplemented, or not, with a blend of LPS (25 µg/mL) and PHA (5 µg/mL), or ionomycin (100 ng/mL) and PMA (100 ng/mL). Results are shown below.

	Sw TNF-α (pg/mL)			
Stimulus	**Cell type**	**24 hrs.**	**48 hrs.**	**72 hrs.**
Neat	WB cells	ND	ND	ND
LPS + PHA	WB cells	212	181	168
PMA + ionomycin	WB cells	27,040	17,080	12,520

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