DRG® Interleukin-18 (rat) ELISA (EIA-4002)

Revised 15 June 2005 **For Veterinary Use Only**

INTRODUCTION

IL-18, also known as Interferon-gamma Inducing Factor (IGIF), is a cytokine with Mr=18 kDa (157 amino acid residues) produced by macrophages and monocytes, Kuppfer cells, keratinocytes, intestinal epithelial cells, osteoblasts, mouse diencephalon, and adrenal cortical cells of reserpine-treated rats. IL-18 is synthesized as an inactive precursor molecule with Mr=24 kDa which lacks a signal peptide. The IL-18 precursor is cleaved by IL-1 converting enzyme (ICE, Caspase-1), producing the bioactive, mature form. Only the mature, 18 kDa, form of IL-18 is secreted. Cells that respond to IL-18 include Th1-type cells and NK cells.

IL-18 exerts several effects on Th1-like cells. IL-18 stimulates Th1 cell proliferation, Fas ligand expression and IL-2R alpha chain expression.

IL-18 also works in combination with IL-12 to induce the production of interferon-gamma, GM-CSF, and IL-2 by Th1-type cells. Standard bioassays for mIL-18 measure dose dependent interferon-gamma production by IL-18 target cells, such as mouse IL-18 receptor transfected KG-1 cells (human myelomonocyte: ATCC CCL246).

Immunomodulatory pathways, which include IL-18 stimulation of interferon-gamma production, are under investigation. Interferon-gamma production by Th1-type cells and NK cells is important in many immune functions, including defense against viral and parasitic infections, enhancement of NK activity, activation of macrophages, enhancement of B cell function including B cell maturation, proliferation and immunoglobulin secretion, enhancement of MHC class I and class II antigen expression, and inhibition of osteoclast activation.

IL-18 is implicated as a mediator of septic shock and tissue injury in response to inflammation, and is implicated in some forms of diabetes.

Septic shock, induced by challenge with lipopolysaccharides and liver damage found in mice following treatment with *Propionibacterium acnes*, can be abrogated by administration of anti-IL-18 antibodies. IL-18 is overexpressed in autoimmune non-obese diabetic mice (NOD).

INTENDED USE

The DRG Rat Interleukin-18 (rIL-18) ELISA is to be used for the *in vitro* quantitative determination of IL-18 in rat serum, EDTA plasma, buffered solution, or cell culture medium. The assay will recognize both natural and recombinant rIL-18.

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 rIL-18 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A polyclonal antibody specific for rIL-18 has been coated onto the wells of the microtiter strips provided. Samples, including standards of known rIL-18 content, control specimens, and unknowns, are pipetted into these wells.

During the first incubation, the rIL-18 antigen binds to the immobilized (capture) antibody on one site. After washing, a biotinylated monoclonal antibody specific for rIL-18 is added. During the second incubation, this antibody binds to the immobilized rIL-18 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 rIL-18 present in the original specimen.

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REAGENTS PROVIDED:

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

Reagent	***96 Test Kit***	***192 Test Kit***
rIL-18 Standard, recombinant baculovirus rIL-18, 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
rIL-18 High and Low Control, recombinant baculovirus rIL-18, lyophilized. Refer to vial label for reconstitution volume and range.	2 vials	4 vials
rIL-18 Antibody-Coated Wells, 96 wells per plate.	1 plate	2 plates
rIL-18 Biotin Conjugate (Biotin-labeled anti-IL-18). Contains 8 mM sodium azide; 11 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.

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 >1000 pg/mL should be diluted with *Standard Diluent Buffer* for serum/plasma samples and with corresponding medium for cell culture samples.
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 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 rIL-18 standard was calibrated against a highly purified recombinant baculovirus protein.

A. Reconstitution and Dilution of rIL-18 Standard

Note: Either glass or plastic tubes may be used for standard dilutions.
1. Reconstitute standard to 5,000 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. It is recommended that standard be used within 1 hour of reconstitution.

2. Add 0.1 mL of the reconstituted standard to a tube containing 0.400 mL *Standard Diluent Buffer*. Label as 1000 pg/mL rIL-18. Mix.
3. Add 0.250 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 500, 250, 125, 62.5, 31.3 and 15.6 pg/mL rIL-18.
4. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution of rIL-18 Standard

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

Standard:	Add:	Into:
1000 pg/mL	Prepare as described in Step 2.	
500 pg/mL	0.250 mL of the 1000 pg/mL std.	0.250 mL of the Diluent Buffer
250 pg/mL	0.250 mL of the 500 pg/mL std.	0.250 mL of the Diluent Buffer
125 pg/mL	0.250 mL of the 250 pg/mL std.	0.250 mL of the Diluent Buffer
62.5 pg/mL	0.250 mL of the 125 pg/mL std.	0.250 mL of the Diluent Buffer
31.3 pg/mL	0.250 mL of the 62.5 pg/mL std.	0.250 mL of the Diluent Buffer
15.6 pg/mL	0.250 mL of the 31.3 pg/mL std.	0.250 mL of the Diluent Buffer
0 pg/mL	0.250 mL of the Diluent Buffer	An empty tube

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 uL 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 uL solution	2 mL
4	40 uL solution	4 mL
6	60 uL solution	6 mL
8	80 uL solution	8 mL
10	100 uL solution	10 mL
12	120 uL 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. Add 100 uL of the *Standard Diluent Buffer* to zero wells. Well(s) reserved for chromogen blank should be left empty.
3. Add 100 uL of standards, samples or controls to the appropriate microtiter wells. (See **REAGENT PREPARATION AND STORAGE**, Section B.)
4. Add 50 uL of *Incubation Buffer* to zero wells and to the wells containing standards and serum/plasma samples, or 50 uL of *Standard Diluent Buffer* to the wells containing cell culture samples and controls. Well(s) reserved for chromogen blank should be left empty. Tap gently on the side of the plate to mix.
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 uL of biotinylated anti-IL-18 (*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 uL 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 uL 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 uL 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 uL 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 rIL-18 concentrations for unknown samples and controls from the standard curve plotted in step 17. (Samples producing signals greater than that of the highest standard (1000 pg/mL) should be diluted in *Standard Diluent Buffer* for serum/plasma samples or corresponding medium for cell culture samples 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 1000 pg/mL rIL-18.

Standard rIL-18 (pg/mL)	Optical Density (450 nm)
0	0.067
	0.071
15.6	0.150
	0.156
31.3	0.229
	0.208
62.5	0.372
	0.380
125	0.651
	0.619
250	1.103
	1.154
500	1.956
	1.928
1000	3.064
	2.991

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 >1000 pg/mL with *Standard Diluent Buffer* for serum/plasma samples and with corresponding medium for cell culture samples; 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 rIL-18 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 rIL-18 is <4 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 rIL-18 concentration were assayed in replicates of 22 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	93	387	877
SD	3.4	17.3	30.4
%CV	3.7	4.5	3.5

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision
Samples were assayed 22 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	89	386	850
SD	6.0	17.1	38.4
%CV	6.7	4.4	4.5

SD = Standard Deviation
CV = Coefficient of Variation

LINEARITY OF DILUTION
Rat serum and cell culture samples were serially diluted in *Standard Diluent Buffer* or *RPMI containing 1% fetal calf serum*, respectively, over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded an average correlation coefficient of 0.99.

Dilution	Serum			Cell Culture		
	Measured (pg/mL)	**Expected (pg/mL)**	**% Expected**	**Measured (pg/mL)**	**Expected (pg/mL)**	**% Expected**
Neat	180	-	-	194	-	-
1/2	87	90	97	90	97	93
1/4	43	45	96	44	48.5	91
1/8	28	22.5	124	25.7	24.3	105
1/16	14	11	124	13.5	12.1	111

RECOVERY
The recovery of rIL-18 added to rat serum averaged 90%. The recovery of rIL-18 added to EDTA plasma averaged 95%. The recovery of rIL-18 added to tissue culture medium containing 1% fetal calf serum averaged 101%, while the recovery of rIL-18 added to tissue culture medium containing 10% fetal calf serum averaged 97%.

SPECIFICITY
Buffered solutions of a panel of substances at 100 ng/mL were assayed with the BioSource International, Inc. rIL-18 kit. The following substances were tested and found to have no cross-reactivity: human IL-18, rat IL-1b, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, MIP-2, TNF-a, CINC-2b, VEGF, GM-CSF; mouse IL-18. Both *E. Coli* and baculovirus derived rIL-18 were detectable with this kit.

EXPECTED VALUES
Four pools of rat serum and one pool of rat EDTA plasma were evaluated in this assay. The following concentrations were detected:
Pool serum 1 : 23 pg/mL
Pool serum 2 : 51 pg/mL
Pool serum 3 : 149 pg/mL
Pool serum 4 : 7 pg/mL
Pool EDTA plasma : 6.5 pg/mL

STIMULATION PROTOCOLS

Cell culture supernatants were evaluated in this assay.

Rat Whole Blood (WB) cells were cultured in RPMI for 24, 48 or 72 hours either without stimulus or with a blend of LPS (25 ug/mL) and PHA (5 ug/mL), or with a blend of ionomycin (100 ng/mL) and PMA (100 ng/mL). Results are shown below.

		rIL-18 (pg/mL)		
Stimulus	**Cell type**	***24 hrs.***	***48 hrs.***	***72 hrs.***
None	WB cells	<4	<4	<4
LPS + PHA	WB cells	8	13	20
PMA + ionomycin	WB cells	8	22	27

Rat splenocytes were cultured at different cellular concentrations in RPMI supplemented with 5% FCS for 24, 48, 72 or 96 hours with a blend of LPS (25 ug/mL) and PHA (5 ug/mL). Results are shown below:

		rIL-18 (pg/mL)			
Cell concentration	**Cell type**	***24 hrs.***	***48 hrs.***	***72 hrs.***	***96 hrs.***
0.25 x 106 cells/mL	Splenocytes	17.4	27	36	45
0.8 x 106 cells/mL	Splenocytes	49	ND	75	89
2.5 x 106 cells/mL	Splenocytes	167	212	238	220

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