DRG® Mouse IL-2 ELISA (EIA-3155 and EIA-3163)

Revised 29 June 2005 **For Veterinary Use Only**

INTRODUCTION

Interleukin-2 (IL-2), also known as T Cell Growth Factor, is a 15.5 kDa glycoprotein secreted primarily by activated T lymphocytes. The protein consists of 133 amino acids and is the product of several post translational processing steps. Transcription and release of synthesized IL-2 is stimulated by antigen or mitogen activation of mature T lymphocytes. IL-2 binds to cell surface receptors and promotes the clonal expansion of antigen-specific effector T cells. IL-2 is a T cell differentiation factor, able to induce the production of other lymphokines, such as IL-4 and interferon-ã. IL-2 promotes the growth of B cells and induction of immunoglobulin secretion. IL-2 stimulates J chain synthesis which leads to assembly and secretion of IgM. Myeloid cell populations such as macrophage precursors and primary peripheral blood monocytes express IL-2 receptors. The binding of IL-2 will cause, in these cells, proliferation, differentiation and enhancement of cytolytic activity (1,2).

The IL-2/IL-2 receptor system appears to play a pathogenic role in several clinical syndromes in humans. The Tax gene product derived from HTLV-I causes a dysregulation of cellular gene expression that may underlie development of Adult T Cell leukemia. One of the genes that is dysregulated is the á subunit of IL-2 receptor (2,3). The genetic defect in X-linked severe combined immunodeficiency (X-SCID) has been mapped to the IL-2 receptor ã gene locus (4). Selective agonists and antagonists of the IL-2 receptor system are the potential targets of therapeutic modalities.

INTENDED USE

The DRG Mouse Interleukin-2 (mIL-2) ELISA is to be used for the *in vitro* quantitative determination of mIL-2 in mouse serum, plasma, buffered solution, or cell culture medium. The assay will recognize both natural and recombinant mIL-2.

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 mIL-2 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for mIL-2 has been coated onto the wells of the microtiter strips provided. Samples, including standards of known mIL-2 content, control specimens, and unknowns, are pipetted into these wells, followed by the addition of a biotinylated monoclonal second antibody.

During the first incubation, the mIL-2 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 mIL-2 present in the original specimen.

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REAGENTS PROVIDED

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

Reagent	***96 Test Kit***	***192 Test Kit***	***480 Test Kit***
mIL-2 Standard, recombinant mIL-2. 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
mIL-2 High and Low Control, recombinant mIL-2, lyophilized. Refer to vial label for quantity and reconstitution volume. Once reconstituted, aliquot and store at -20°C or below. Avoid repeated freeze-thaw cycles.	2 vials	2 vials	4 vials
mIL-2 Antibody-Coated Wells, 96 wells per plate.	1 plate	2 plates	5 plates
mIL-2 Biotin Conjugate (Biotin-labeled anti-IL-2). 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	4	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 - 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.
9. 37°C incubator.

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*.
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. If your automated plate washer allows, 30 second soak cycles should be programmed into the wash cycle.

REAGENT PREPARATION AND STORAGE

A. Reconstitution and Dilution of mIL-2 Standard

One microgram of DRG recombinant mouse IL-2 equals 330,000 arbitrary units of WHO reference preparation 93/566 (NIBSC, Hertfordshire, UK, EN6 3QG).

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

1. Reconstitute standard to 10,000 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 0.050 mL of the reconstituted standard to a tube containing 0.450 mL *Standard Diluent Buffer*. Label as 1000 pg/mL mIL-2. Mix.
3. Add 0.300 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 500, 250, 125, 62.5, 31.2, and 15.6 pg/mL mIL-2.
4. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution of mIL-2 Standard

Standard:	Add:	Into:
1000 pg/mL	Prepare as described in Step 2.	
500 pg/mL	0.150 mL of the 1000 pg/mL std.	0.150 mL of the Diluent Buffer
250 pg/mL	0.150 mL of the 500 pg/mL std.	0.150 mL of theDiluent Buffer
125 pg/mL	0.150 mL of the 250 pg/mL std.	0.150 mL of the Diluent Buffer
62.5 pg/mL	0.150 mL of the 125 pg/mL std.	0.150 mL of the Diluent Buffer
31.2 pg/mL	0.150 mL of the 62.5 pg/mL std.	0.150 mL of the Diluent Buffer
15.6 pg/mL	0.150 mL of the 31.2 pg/mL std.	0.150 mL of the Diluent Buffer
0 pg/mL	0.150 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. 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. Add 50 µL of the *Standard Diluent Buffer* to zero wells. Well(s) reserved for chromogen blank should be left empty.
3. Add 50 µL of standards, samples or controls to the appropriate microtiter wells. (See **REAGENT PREPARATION AND STORAGE**, Section B.)
4. Pipette 50 µL of biotinylated anti-IL-2 (*Biotin Conjugate*) solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.
5. Cover plate with *plate cover* and incubate for **2 hours at 37°C**.
6. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
7. 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.)
8. Cover plate with the *plate cover* and incubate for **30 minutes 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 of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.
11. 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.

12. 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.
13. 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*.
14. 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.
15. Read the mIL-2 concentrations for unknown samples and controls from the standard curve plotted in step 14. (Samples producing signals greater than that of the highest standard (1000 pg/mL) should be diluted in *Standard Diluent Buffer* and re-analyzed, 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 mIL-2.

Standard mIL-2 (pg/mL)	Optical Density (450 nm)
0	0.032
	0.034
15.6	0.070
	0.071
31.2	0.108
	0.112
62.5	0.206
	0.231
125	0.444
	0.454
250	0.853
	0.911
500	1.533
	1.594
1000	2.365
	2.386

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*; 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 mIL-2 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 mIL-2 is <8 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 mIL-2 concentration were assayed in replicates of 32 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	114.4	391.7	771.9
SD	5.5	10.2	32.8
%CV	4.8	2.6	4.3

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision
Samples were assayed 30 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	118.5	365.4	749.7
SD	6.7	16.3	41.7
%CV	5.6	4.4	5.5

SD = Standard Deviation
CV = Coefficient of Variation

LINEARITY OF DILUTION
Mouse serum and tissue culture medium containing 1% fetal calf serum were spiked with mIL-2 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.

	Serum			Cell Culture		
Dilution	Measured (pg/mL)	Expected (pg/mL)	% Expected	Measured (pg/mL)	Expected (pg/mL)	% Expected
neat	904	--	--	888	--	--
1/2	492	452	109	425	444	96
1/4	246	226	109	220	222	99
1/8	110	113	97	111	111	100
1/16	54	57	95	58	56	104

1/32	26	28	93	28	28	100

RECOVERY

The recovery of mIL-2 added to normal mouse serum averaged 89% (range: 85% to 106%). The recovery of mIL-2 added to mouse plasma (heparin) averaged 89% (range: 82% to 97%). The recovery of mIL-2 added to tissue culture medium containing 1% fetal calf serum averaged 93%, while the recovery of mIL-2 added to tissue culture medium containing 10% fetal calf serum averaged 99%.

PARALLELISM

Natural mIL-2 was serially diluted in *Standard Diluent Buffer*. The optical density of each dilution was plotted against the standard curve.

Parallelism between the natural and recombinant protein was demonstrated by the figure below and indicated that the standard accurately reflects natural mIL-2 content in samples.

SPECIFICITY

Buffered solutions of a panel of substances at 10,000 pg/mL were assayed with the DRG mIL-2 kit. The following substances were tested and found to have no cross-reactivity: human IL-2, IL-5, IL-12, GM-CSF, RANTES; mouse IL-1b, IL-3, IL-4, IL-6, IL-10, IFN-y, MCP-1, TNF-a; rat IL-1a, IL-1b, IL-2, IL-4, IL-10, IFN-y, TNF-a.

EXPECTED VALUES

Sixteen sera, sixteen plasma (heparin) samples were evaluated in this assay. All samples measured <15.6 pg/mL (the lowest mIL-2 standard).

Mouse splenocytes were cultured under the following conditions and the culture supernatants were assayed for mIL-2 released.

1. Con-A (5 µg/mL) 6 hr: 605 pg/mL
2. PMA (50 ng/mL), Ionophore (250 ng/mL) 6 hr: 555 pg/mL
3. PMA (50 ng/mL), Ionophore (250 ng/mL) 24 hr: 2724 pg/mL
4. LPS (1 µg/mL) 24 hr: 827 pg/mL

REFERENCES

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