DRG® IL-12 p70 (rat) ELISA (EIA-4001)

REVISED 15 JUNE 2005 **FOR VETERINARY USE ONLY**

1 INTRODUCTION

Interleukin-12 (IL-12) is a heterodimeric cytokine that plays a central role in promoting type 1 T helper cell (Th1) responses and thus, cell-mediated immunity. Its active form is a disulfide-linked, 70 kDa (p70) glycoprotein composed of 40 kDa (p40) and 35 kDa (p35) subunits. The individual p40 and p35 subunits show no IL-12 activity, although p40 does exist as a dimer that has been shown to bind the IL-12 receptor and act as an IL-12 antagonist. IL-12 is produced by phagocytic cells, antigen-presenting cells and B lymphocytes in response to bacteria or intracellular parasites. IL-12 acts on T and natural killer (NK) cells inducing proliferation, enhancement of cell-mediated cytotoxicity and production of cytokines, particularly gamma-interferon (IFN-γ). IL-12 and IL-12-induced IFN-γ are considered critical in early immune responses. If both are present during early T cell expansion in response to antigen, Th1 cell generation is favored and generation of Th2 cells is inhibited (1,2).
Human and rat IL-12 share 64% and 58% amino acid sequence homology in their p40 and p35 subunits, respectively. IL-12 apparently shows species specificity, with human IL-12 reportedly showing minimal activity in the rat system. The p40 subunit of IL-12 has been shown to have extensive amino acid sequence homology to the extracellular domain of the human IL-6 receptor while the p35 subunit shows distant but significant sequence similarity to IL-6 and G-CSF. These observations have led to the suggestion that IL-12 might have evolved from a cytokine/soluble receptor complex.
Both p40 and p35 are LPS-inducible in monocytes following IFN-gamma pre-treatment. Although each subunit is independently controlled, the regulated expression of p35 determines the level of active IL-12 protein (3). Free p35 has not been detected in supernatant solutions of cultured cells expressing only p35 or both p35 and p40 mRNAs. In contrast, p40 is secreted in excess of IL-12 in cells expressing both p35 and p40 mRNAs.
IL-12 activity is mediated through a high-affinity receptor composed of two subunits, designated beta 1 and beta 2. Of these two subunits, beta 2 is more restricted in its distribution, and regulation of its expression is likely a central mechanism by which IL-12 responsiveness is controlled. Studies with neutralizing anti-IL-12 antibodies and IL-12-deficient mice have suggested that endogenous IL-12 plays an important role in the normal host's defense against infection. IL-12 appears also to play an important role in the genesis of certain forms of immunopathology. Inhibition of IL-12 synthesis or activity may be beneficial in diseases associated with pathologic Th1 responses, such as multiple sclerosis or Crohn's disease. On the other hand, administration of recombinant IL-12 may have utility in the treatment of diseases associated with pathologic Th2 responses such as allergic disorders and asthma (4).

2 INTENDED USE

The DRG Rat Interleukin-12 p70 [IL-12 p70 (rat)] ELISA is to be used for the in vitro quantitative determination of IL-12 in rat serum, EDTA plasma, buffered solution, or cell culture medium. The assay will exclusively recognize both natural and recombinant rIL-12 heterodimer.

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

3 PRINCIPLE OF THE METHOD

The DRG IL-12 p70 (rat) kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for rIL-12 p35 has been coated onto the wells of the microtiter strips provided. Samples, including standards of known rIL-12 content, control specimens, and unknowns, are pipetted into these wells.
During the first incubation, the rIL-12 antigen binds to the immobilized (capture) antibody on one site. After washing, a biotinylated monoclonal antibody specific for rIL-12 p40 is added. During the second incubation, this antibody binds to the immobilized rIL-12 heterodimer 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-12 present in the original specimen.

4 REAGENTS PROVIDED

Note: Store all reagents at 2 - 8°C.

Reagent	96 Test Kit
rIL-12 p70 Standard, recombinant baculovirus rIL-12, lyophilized. Refer to vial label for quantity and reconstitution volume.	2 vials
Standard Diluent Buffer. Contains 8 mM sodium azide; 25 mL per bottle.	1 bottle
Incubation Buffer. Contains 8 mM sodium azide; 11 mL per bottle.	1 bottle
rIL-12 p70 High and Low Control, recombinant baculovirus rIL-12, lyophilized. Refer to vial label for reconstitution volume and range.	2 vials
rIL-12 Antibody-Coated Wells, 96 wells per plate.	1 plate
rIL-12 Biotin Conjugate (Biotin-labeled anti-IL-12). Contains 8 mM sodium azide; 11 mL per bottle.	1 bottle
Streptavidin-Peroxidase (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.	4

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.

5 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 H_2O.
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.

6 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. Micr**otiter 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 >500 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.

7 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.

8 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.

9 REAGENT PREPARATION AND STORAGE

The rIL-12 standard was calibrated against a highly purified baculovirus recombinant protein expressed in S¦9 cells.

9.1 Reconstitution and Dilution of rIL-12 p70 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 0.05 mL of the reconstituted standard to a tube containing 0.450 mL Standard Diluent Buffer. Label as 500 pg/mL rIL-12. Mix.
3. Add 0.250 mL of Standard Diluent Buffer to each of 6 tubes labeled 250, 125, 62.5, 31.3, 15.6 and 7.8 pg/mL rIL-12.
4. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

9.2 Dilution of rIL-12 p70 Standard

Standard:	Add:	Into:
500 pg/mL	Prepare as described in Step 2.	
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
7.8 pg/mL	0.250 mL of the 15.6 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.

9.3 Storage and Final Dilution of Streptavidin-HRP

1. Dilute 10 mL 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.

9.4 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.

10 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 µL of the Standard Diluent Buffer to zero wells.
   Well(s) reserved for chromogen blank should be left empty.
3. Add 100 µL of standards, samples or controls to the appropriate microtiter wells. (See REAGENT PREPARATION AND STORAGE, Section B.)

4. Add 50 µL of Incubation Buffer to zero wells and to the wells containing standards, controls and serum/plasma samples,
   or 50 µL of Standard Diluent Buffer to the wells containing cell culture samples.
   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 µL of biotinylated anti-IL-12 (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. (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-12 concentrations for unknown samples and controls from the standard curve plotted in step 17. (Samples producing signals greater than that of the highest standard (500 pg/mL) should be diluted in Standard Diluent Buffer for serum/plasma samples or corresponding medium for cell culture samples and re-analyzed, multiplying the concentration found by the appropriate dilution factor.

10.1 Typical Data

The following data were obtained for the various standards over the range of 0 to 500 pg/mL rIL-12.

Standard rIL-12 (pg/mL)	Optical Density (450 nm)
0	0.063 0.070
7.8	0.133 0.145
15.6	0.187 0.203
31.3	0.320 0.318
62.5	0.491 0.512
125	0.889 0.877
250	1.569 1.493
500	2.584 2.702

11 LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 500 pg/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain.
Dilute samples >500 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-12 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.

12 PERFORMANCE CHARACTERISTICS

12.1 Sensitivity

The minimum detectable dose of rIL-12 is <2.5 pg/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times.

12.2 Precision

12.2.1 Intra-Assay Precision

Samples of known rIL-12 concentration were assayed in replicates of 22 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	47	169	416
SD	1.8	5.1	13.1
%CV	3.8	3.0	3.2

SD = Standard Deviation CV = Coefficient of Variation

12.2.2 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)	49	166	392
SD	2.4	5.4	15.7
%CV	4.9	3.2	4.0

12.3 LINEARITY OF DILUTION

Rat serum or cell culture samples containing IL-12 were serially diluted over the range of the assay in Standard Diluent Buffer or RPMI containing 1% fetal calf serum, respectively.
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	367	-		359	-	
1/2	181	184	101	180	179.5	100
1/4	85	92	92	89.6	89.8	99.8
1/8	44.5	46	97	41.4	45	92
1/16	21.2	23	92	21.3	22.4	95

12.4 RECOVERY

The recovery of rIL-12 added to rat serum averaged 97%.
The recovery of rIL-12 added to tissue culture medium containing 1% fetal calf serum averaged 98%, while the recovery of rIL-12 added to tissue culture medium containing 10% fetal calf serum averaged 96%.

12.5 SPECIFICITY

Buffered solutions of a panel of substances at 50 ng/mL were assayed with the DRG IL-12 p70 (rat) kit.
The following substances were tested and found to have no cross-reactivity:
rat IL-1b, IL-2, IL-4, IL-6, IL-10, IL-18, MIP-2, TNF-a, CINC-2b, VEGF, IFN-g;
mouse IFN-g; IL-12, IL-15, IL-18, VEGF;
Swine IL-1b, IL-4, IL-6, IL-8, IL-15, IFN-g, TNF-a and
human IL-12.
A large excess of the p40 subunit of rat IL-12 (100 ng/mL) did not interfere with the quantitation of rat IL-12 p70.
Both E. Coli and baculovirus derived rIL-12 were detectable with this kit.

12.6 EXPECTED VALUES

Twelve pools of rat serum and one pool of EDTA plasma were evaluated in this assay.
The values were below 2.5 pg/mL.

13 REFERENCES

1. Trinchieri, G. (1998) Adv. Immunol. 70:83-243.
2. Trinchieri, G. (1997) Curr. Opin. Immunol. 9(1):17-23.
3. Hayes, M.P., et al. (1995) Blood 86(2):646-650.
4. Gately, M.K., et al. (1998) Annu. Rev. Immunol. 16:495-521.