DRG® Interleukin-4 (Porcine) ELISA (EIA-4091)

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

1. NAME AND INTENDED USE

The DRG Interleukin-4 (porcine)(swIL-4) ELISA is to be used for the in vitro quantitative determination of swIL-4 in swine serum, buffered solution, or cell culture medium. The assay recognizes both recombinant and natural swine IL-4.
This kit has been configured for research use only and is not to be used in diagnostic procedures.

Read entire protocol before use.

2. INTRODUCTION

Interleukin-4 (IL-4) is a 15-19 kDa glycoprotein produced by the Th2 sub-type of CD4+ T-lymphocytes and by mast cell precursors. IL-4 down regulates the production of IFN-γ by Th1 CD4+ T-lymphocytes, induces the proliferation of thymocytes and mature T-lymphocytes but blocks the IL-2 induced proliferation of peripheral T-cells as well as the production of IL-2 dependent LAK cells. On B-cells, IL-4 has a growth factor activity mediated via the production of soluble CD-23, and a differentiation activity leading to the production of IgE, IgM and IgG1.
On monocytes, IL-4 induces an increased number of histocompatibility class II antigens and CD-23 receptors but inhibits the expression of IgG receptors. IL-4 blocks the production of IL-1, IL-6, TNF-α, PGE2, G-CSF and stimulates the production of M-CSF and G-CSF by the monocytes. IL-4 has also an action on eosinophils by increasing the expression of CD-23 and inhibiting the expression of IgG receptors.
Through its pleiotropic activity, IL-4 is a key cytokine in the immune network that shows anti-inflammatory properties and is probably involved in mechanisms of allergy.

3. PRINCIPLE OF THE TEST

The DRG swIL-4 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA).
A monoclonal antibody specific for swIL-4 has been coated onto the wells of the microtiter strips provided. Samples, including standards of known swIL-4 content, control specimens, and unknowns, are pipetted into these wells, followed by the addition of a second monoclonal biotinylated antibody.
During the first incubation, the swIL-4 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 swIL-4 present in the original specimen.

4. WARNINGS AND PRECAUTIONS FOR USERS

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.
Proclin® 300 is toxic.
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.

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. The swIL-4 ELISA kit may be used to measure IL-4 in serum, buffered solution and cell culture samples.
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. Do not allow wells to dry out.
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 or local authorities when handling and disposing infectious agents.

5. KIT COMPONENTS

Reagents	Quantity (192 tests)	Preparation and Storage
Swine IL-4 Standard, purified recombinant swIL-4 expressed in *E. coli*. Refer to vial label for quantity and reconstitution volume.	4 vials	Lyophilized; 2 – 8°C
Standard Diluent Buffer Contains 8 mM sodium azide.	2 x 25 mL	2 – 8°C
Swine IL-4 Antibody-Coated Wells 96 wells per plate.	2 plates	2 – 8°C
swIL-4 High and Low Control recombinant swIL-4 in tissue culture matrix. Refer to vial label for reconstitution volume and range. Once reconstituted, aliquot and store at –20°C or below. Avoid repeated freeze-thaw cycles.	2 vials	lyophilized 2 – 8°C
Swine IL-4 Biotin Conjugate (Biotin-labeled anti-IL-4). Contains 8 mM sodium azide.	2 x 6 mL	2 – 8°C
Streptavidin-Peroxidase (HRP), (100x) Contains 1.7 mM thymol.	2 x 0.125 mL	Concentrate; 2 – 8°C
Streptavidin-Peroxidase (HRP) Diluent. Contains 1.7 mM thymol and 0.05% Proclin® 300.	1 x 25 mL	2 – 8°C
Wash Buffer (25x)	1 x 100 mL	Concentrate; 2 – 8°C
Stabilized Chromogen Tetramethylbenzidine (TMB).	1 x 25 mL	2 – 8°C
Stop Solution	1 x 25 mL	2 – 8°C
Plate Covers, adhesive strips.	4	

6. MATERIALS REQUIRED BUT NOT SUPPLIED

Microtiter plate reader capable of measurement at or near 450 nm.

- Calibrated adjustable precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable for large assays.)
- Deionized or distilled H_2O.
- Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
- Graph paper: linear (Cartesian), log-log, or semi-log, as desired.
- Glass or plastic tubes for diluting and aliquoting standard.
- Absorbent paper towels.
- Calibrated beakers and graduated cylinders in various sizes.

7. SPECIMEN COLLECTION AND PREPARATION OF SAMPLES

Samples should be collected in pyrogen/endotoxin-free tubes. The swIL-4 ELISA kit may be used to measure IL-4 in serum, buffered solution and cell culture samples.
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.
When possible, avoid use of badly hemolyzed or lipemic sera. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.
Samples that are >1000 pg/mL should be diluted with *Standard Diluent Buffer*.

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 PROCEDURE. 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

Reconstitution and Dilution of swIL-4 Standard

The swIL-4 standard was calibrated against the mass of a highly purified, *E. coli*-expressed recombinant protein produced at DRG Instruments.
Note: Either glass or plastic tubes may be used for standard dilutions.

1. Reconstitute standard to 5000 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 0.100 mL of the reconstituted standard to a tube containing 0.400 mL Standard Diluent Buffer. Label as 1000 pg/mL swIL-4. Mix.
3. Add 0.200 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 500, 250, 125, 62.5, 31.3 and 15.6 pg/mL swIL-4.
4. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

Dilution of swIL-4 Standard

Standard:	Add:	Into:
1000 pg/mL	Prepare as described in Step 2.	
500 pg/mL	0.200 mL of the 1000 pg/mL std.	0.200 mL of the Diluent Buffer
250 pg/mL	0.200 mL of the 500 pg/mL std.	0.200 mL of the Diluent Buffer
125 pg/mL	0.200 mL of the 250 pg/mL std.	0.200 mL of the Diluent Buffer
62.5 pg/mL	0.200 mL of the 125 pg/mL std.	0.200 mL of the Diluent Buffer
31.3 pg/mL	0.200 mL of the 62.5 pg/mL std.	0.200 mL of the Diluent Buffer
15.6 pg/mL	0.200 mL of the 31.3 pg/mL std.	0.200 mL of the Diluent Buffer
0 pg/mL	0.200 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.

10. 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.

11. 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.

12. ASSAY PROCEDURE

General Remarks

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.

Assay Procedure

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 9.2.)
4. Pipette 50 µL of *biotinylated anti-IL-4* (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 room temperature**.
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 9.3.)
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.

Calculation

1. 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.
2. Read the swIL-4 concentrations for unknown samples and controls from the standard curve plotted in step 1. (Samples producing signals greater than that of the highest standard (1000 pg/mL) should be further diluted in *Standard Dilutent 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 swIL-4.

Standard swIL-4 (pg/mL)	Optical Density (450 nm)
0	0.028 0.016
15.6	0.079 0.073
31.3	0.134 0.137
62.5	0.244 0.263
125	0.439 0.487
250	0.832 0.842
500	1.547 1.561
1000	2.619 2.681

13. EXPECTED VALUES

Each laboratory must establish its own normal values. For guidance, the mean of 28 normal swine sera was <2 pg/mL. Cell culture supernatants were evaluated in this assay. Swine Whole Blood (WB) as well as lymph node (LN) cells were cultured in RPMI supplemented with 5% FCS for 24, 48 or 72 hours either without stimulation, or with PHA (5 µg/mL), or with a blend of LPS (25 µg/ mL) and PHA (5 µg/mL), or with a blend of ionomycin (100 ng/mL) and PMA (100 ng/mL).
Results are shown below (NT= not tested).

Levels of IL-4 released from cultured swine cells

		Swine IL-4 (pg/mL)		
Stimulation Condition	**Cell type**	**24 hrs**	**48 hrs.**	**72 hrs.**
None	WB cells	<2	<2	<2
LPS + PHA	WB cells	148	NT	NT
PMA + ionomycin	WB cells	259	225	208
PHA	LN cells	NT	205	NT
PMA + ionomycin	LN cells	NT	104	NT

14. PERFORMANCE CHARACTERISTICS

Sensitivity

The minimum detectable dose of swIL-4 is 2 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 swIL-4 concentration were assayed in replicates of 22 to determine precision within an assay.

Sample	Sample 1	Sample 2	Sample 3
Mean [pg/ml]	48	394	781
S.D.	1.8	12.5	25
%CV	3.7	3.2	3.2

SD = Standard Deviation
CV = Coefficient of Variation

Inter-Assay Precision

Samples were assayed 22 times in multiple assays to determine precision between assays.

Sample	Sample 1	Sample 2	Sample 3
Mean [pg/ml]	50	385	766
S.D.	2.4	18.8	28.5
%CV	4.8	4.9	3.7

Linearity of Dilution

Cell culture or serum samples containing swIL-4 were 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.

Dilution	Cell Culture Fluid			Serum		
	Measured (pg/mL)	Expected (pg/mL)	% Expected	Measured (pg/mL)	Expected (pg/mL)	% Expected
neat	984	-	-	615	-	-
1/2	510	492	103.7	305	308	99.0
1/4	256	246	104.1	149	154	96.8
1/8	128	123	104.1	69	77	89.6
1/16	67	62	108.1	Not Done		

Recovery

The recovery of swIL-4 added to swine serum averaged 95%.
The recovery of swIL-4 added to tissue culture medium containing 10% fetal calf serum averaged 92%.

Parallelism

Natural IL-4 was serially diluted in Standard Diluent Buffer. The optical density of each dilution was plotted against the standard curve. Parallelism between the natural IL-4 and the standard protein was demonstrated and indicated that the standard accurately reflects natural IL-4 content in samples.

Specificity

Buffered solutions of a panel of substances at 150 ng/mL were assayed with the DRG swIL-4 kit.
The following substances were tested and found to have no cross-reactivity: human IL-4; mouse IL-4, rat IL-4; swine IL-1β, IL-2, IL-6, IL-8, IL-10, IL-15, TNF-α and IFN-γ.

15. LIMITATIONS OF 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 swIL-4 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.**
- Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the package insert instruction and with adherence to good laboratory practice.
- The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbances.

16. REFERENCES

1. Banchereau J. (1990). Médecine/Science 6:946-953.
2. Miossec P. et al. (1990). Arth. Rheum. 33:1180-1187.
3. Yokota T. et al. (1988). Immunol. Rev. 102:137-187.