Human IL-5 ELISA Kit

For the quantitative determination of human interleukin 5 (IL-5)
concentrations in serum, plasma,
cell culture medium, and urine

Catalogue Number: EL10035

96 tests

FOR LABORATORY RESEARCH USE ONLY
NOT FOR DIAGNOSTIC USE

ANOGEN

2355 Derry Road East, Unit 23
Mississauga, Ontario
CANADA L5S 1V6
Tel: (905) 677-9221 or (877) 755-8324
Fax: (905) 677-0023

Email: info@anogen.ca ♦ Web Site: www.anogen.ca
Secure Online Store: www.anogen.net

S7.5 (03) IL-5

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INTENDED USE

This Human Interleukin 5 ELISA Kit is to be used for the *in vitro* quantitative determination of human interleukin 5 (IL-5) concentrations in serum, plasma, cell culture medium, and urine. This kit is intended for LABORATORY RESEARCH ONLY and is not to be used for diagnostic or therapeutic purposes.

INTRODUCTION

Human Il-5 is a 135 amino acid (aa) polypeptide with a predicted mass of 12.5 kDa. It is secreted by a restricted number of mesenchymal cell types. In its native state, mature IL-5 is synthesized as a 115 aa, highly glycosylated 22 kDa monomer that forms a 40-50 kDa disulfide-linked homodimer. Although the content of carbohydrate is high, carbohydrate is not needed for bioactivity. Monomeric IL-5 has no activity and requires a homodimer for function, which is in contrast to the receptor-related IL-3 and GM-CSF cytokines that exist only as monomers. Just as one IL-3 and GM-CSF monomer binds to one receptor, one IL-5 homodimer is able to engage only one IL-5 receptor. It has been suggested that IL-5 (as a dimmer) undergoes a general conformational change after binding to one receptor molecule and this change precludes binding to a second receptor. Human and mouse mature IL-5 are 71% identical at the aa level. While mouse IL-5 is highly active on human cells, human IL-5 is only marginally active on mouse cells.

Although many cells contribute to inflammation, eosinophils are noted for their contribution to late phase allergic-type disorders. Eosinophils make up less than 10% of the circulation leukocyte population, yet they are known to be extremely important in the inflammatory response to allergic and parasitic agents. When activated within tissues, eosinophils release highly basic preformed mediators such as eosinophil peroxidase and major basic protein. These substances are toxic to parasites and damaging to the surrounding tissue functionality including smooth muscle constriction, vascular permeability and superoxide-mediated tissue destruction. While eosinophils have been associated with these inflammatory reactions, the soluble mediators that influence eosinophil availability and function have only recently been identified. Interleukin 5 (IL-5) along with the chemokine eotaxin are key players in the coordination of the eosinophil-based inflammatory response.

The receptor for IL-5 consists of a ligand binding alpha-subunit and a non-ligand binding (common) signal transducing beta-subunit that is shared by the receptors for IL-3 and GM-CSF.

The kinetics of IL-5 binding is still under investigation. Assuming equality with the IL-3 system, (homodimeric) IL-5 binds noncovalently to one IL-5 Rα subunit, which then noncovalently recruits one βc subunit, forming a temporary noncovalently-linked trimer. At this point, a second, newly generated IL-5 R complex can be engaged with the IL-5 Rα subunit from the primary complex by forming a disulfide bond with βc. This is followed by disulfide bonding between the two remaining unlinked receptor components. It is likely that the two βc subunits also become disulphide-linked, creating a functional signal-transducing complex with a stoichiometry of 2:2:2. A newly formed IL-5 R trimer may also link with naturally preformed GM-CSF Rα/βc complexes to form hybrid IL-5

R/GM-CSF R complexes. The function of these complexes and their role with IL-5 activity is currently unknown.

As with many cytokines and growth factors, IL-5 has an approximately 15 aa NLS in the body of the molecule. IL-5 with its receptor can be transported into the nucleus following its binding on the cell surface. It is suggested that STATs, which are associated with the receptor, actually enter the receptor via the IL-5 NLS.

Cells known to express IL-5 include eosinophils, NK cells, TC2 CD8+ T cells, mast cells, CD45+ CD4+ T cells, $\gamma\delta$ T cells and IL-1β activated endothelial cells. IL-5 is best known for its activity on B cells and eosinophils. Relative to cells, IL-5 appears to induce the differentiation of activated conventional B-2 cells. In mice, IL-5 promotes production of IgA, IgE and IgG1.

IL-5 appears to perform a number of functions on eosinophils including growth and differentiation, the down modulation of Mac-1, the upregulation of receptors for IgA and IgG, the stimulation of lipid mediator (leukotriene C4 and PAF) secretion and the induction of granule release. IL-5 may act in an adjunct fashion plays, however, the exact role is unclear. Finally, there is a great deal of interest in the interaction between IL-5 and the CC chemokine eotaxin. Studies suggest that inflammatory-induced and locally produced IL-5 and eotaxin may act on the bone marrow in a cooperative manner.

This IL-5 ELISA is a ready-to-use 3.5-hour solid phase immunoassay readily capable of measuring IL-5 levels in serum, plasma, cell culture medium, and urine in the range of 31.25pg/ml to 1000pg/ml. No cross reactivity with other cytokines tested was observed.

PRINCIPLE OF THE ASSAY

This IL-5 enzyme linked immunosorbent assay (ELISA) applies a technique called a quantitative sandwich immunoassay. The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to IL-5. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated monoclonal antibody preparation specific for IL-5 and incubated. IL-5, if present, will bind and become immobilized by the antibody pre-coated on the wells and then become "sandwiched" by biotin conjugate. The microtiter plate wells are thoroughly washed to remove unbound IL-5 and other components of the sample. In order to quantitatively determine the amount of IL-5 present in the sample, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Avidin is a tetramer containing four identical subunits, each having a high affinity-binding site for biotin. The wells are thoroughly washed to remove all unbound HRP-conjugated Avidin and a TMB (3,3'5,5' tetramethyl-benzidine) substrate solution is added to each well. The enzyme (HRP) and substrate are allowed to react over a short incubation period. Only those wells that contain IL-5, biotin-conjugated antibody, and enzyme-conjugated Avidin will exhibit a change in colour. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm.

LIMITATIONS OF APPLICATION

- The Human IL-5 ELISA kit is not for use in clinical diagnostic procedures, and for laboratory use only.
- Although all manufacturing precautions have been exercised to ensure that this product will be suitable for use with all validated sample types as designated in the product insert, the possibility of interference cannot be excluded due to the variety of proteins that may exist within the sample.
- The Calibrator Diluent selected for the standard curve should be consistent with the assay samples. If the values generated by the samples are greater than the uppermost standard, the samples dilution should be adjusted with the appropriate Calibrator Diluent and the assay should be repeated.

REAGENTS PROVIDED

All reagents provided are stored at 2-8°C. Refer to the expiration date on the label.

	96 tests

1. **IL-5 MICROTITER PLATE** (Part EL35-1)   **96 wells**
    Pre-coated with anti-human IL-5 monoclonal antibody.

2. **BIOTIN CONJUGATE** (Part EL35-2)   **6 mL**
    Anti-human IL-5 monoclonal antibody conjugated to Biotin.

3. **AVIDIN CONJUGATE** (Part EL35-3)   **12 mL**
    Avidin conjugated to horseradish peroxidase.

4. **IL-5 STANDARD** (Part EL35-4)   **2 vials**
    WHO reference Code 90/586. 2000 pg/vial.

5. **CALIBRATOR DILUENT I** (Part EL35-5)   **25 mL**
    Animal serum with buffer and preservative. f*or serum/plasma testing.*

6. **CALIBRATOR DILUENT II** (Part EL35-6)   **25 mL**
    Cell culture medium with calf serum and preservative. *For cell culture medium testing.*

7. **WASH BUFFER (20X)** (Part 30005)   **60 mL**
    20-fold concentrated solution of buffered surfactant.

8. **SUBSTRATE A** (Part EL35-7)   **10 mL**
    Buffered solution with H_2O_2

9. **SUBSTRATE B** (Part 30007)   **10 mL**
    Buffered solution with TMB.

10. **STOP SOLUTION** (Part 30008)   **14 mL**
    2N Sulphuric Acid (H_2SO_4). Caution: Caustic Material!

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Single or multi-channel precision pipettes with disposable tips: 10-100μL and 50-200μL for running the assay.
2. Pipettes: 1 mL, 5 mL 10 mL, and 25 mL for reagent preparation.
3. Multi-channel pipette reservoir or equivalent reagent container.
4. Test tubes and racks.
5. Polypropylene tubes or containers (25 mL).
6. Erlenmeyer flasks: 100 mL, 400 mL, 1 L and 2 L.
7. Microtiter plate reader (450 nm $\pm$ 2nm)
8. Automatic microtiter plate washer or squirt bottle.
9. Sodium hypochlorite solution, 5.25% (household liquid bleach).
10. Deionized or distilled water.
11. Plastic plate cover.
12. Disposable gloves.
13. Absorbent paper.

PRECAUTIONS

1. Do not substitute reagents from one kit lot to another. Standard, conjugate and microtiter plates are matched for optimal performance. Use only the reagents supplied by manufacturer.
2. Allow kit reagents and materials to reach room temperature (20-25°C) before use. Do not use water baths to thaw samples or reagents.
3. Do not use kit components beyond their expiration date.
4. Use only deionized or distilled water to dilute reagents.
5. Do not remove microtiter plate from the storage bag until needed. Unused strips should be stored at 2-8°C in their pouch with the desiccant provided.
6. Use fresh disposable pipette tips for each transfer to avoid contamination.
7. Do not mix acid and sodium hypochlorite solutions.
8. Human serum and plasma should be handled as potentially hazardous and capable of transmitting disease. Disposable gloves must be worn during the assay procedure, since no known test method can offer complete assurance that products derived from human blood will not transmit infectious agents. Therefore, all blood derivatives should be considered potentially infectious and good laboratory practices should be followed.
9. All samples should be disposed of in a manner that will inactivate human viruses.
   Solid Wastes: Autoclave 60 min. at 121°C.
   Liquid Wastes: Add sodium hypochlorite to a final concentration of 1.0%. The waste should be allowed to stand for a minimum of 30 minutes to inactivate the virus before disposal.
10. Substrate Solution is easily contaminated. If bluish prior to use, *do not use*.
11. Substrate B contains 20% acetone, keep this reagent away from sources of heat or flame.
12. If Wash Buffer (20X) is stored at a lower temperature (2-5°C), crystals may form which must be dissolved by warming to 37°C prior to use.

SAMPLE PREPARATION

COLLECTION, HANDLING, AND STORAGE

a) **Cell Culture Medium:** Centrifuge to remove any visible particulate material.

b) **Serum**: Blood should be drawn using standard venipuncture techniques and serum separated from the blood cells as soon as possible. Samples should be allowed to clot for one hour at room temperature, centrifuged for 10 minutes (4°C) and serum extracted.

c) **Plasma:** Blood should be drawn using standard venipuncture techniques and plasma collected using sodium citrate, EDTA, or heparin as an anticoagulant. To ensure optimal recovery and minimal platelet contamination, after collection there must be quick separation of plasma with less than 30 minutes on ice. Centrifuge for 10 minutes (4°C) to remove any particulates.

- Avoid grossly hemolytic, lipidic or turbid samples.

- Serum, plasma, cell culture medium and urine samples to be used within 24-48 hours may be stored at 2-8°C, otherwise samples must stored at -20°C to avoid loss of bioactivity and contamination. Avoid freeze-thaw cycles.
- When performing the assay slowly bring samples to room temperature.
- It is recommended that all samples be assayed in duplicate.
  - DO NOT USE HEAT-TREATED SPECIMENS.
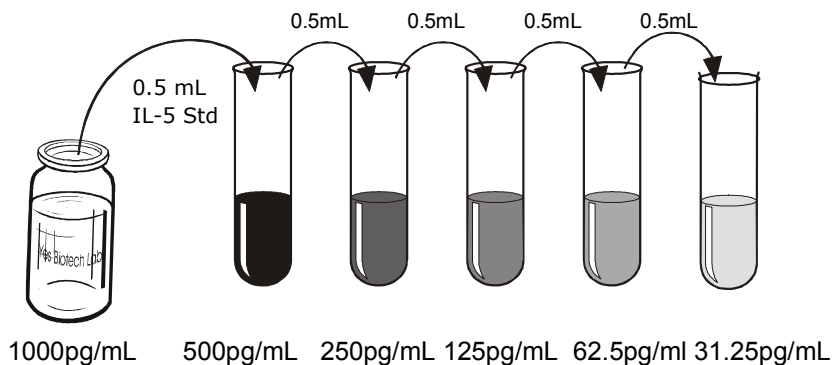
PREPARATION OF REAGENTS

Remove all kit reagents from refrigerator and allow them to reach room temperature (20-25°C). Prepare the following reagents as indicated below. Mix thoroughly by gently swirling before pipetting. Avoid foaming.

1. **Wash Buffer (1X):** Add 60 mL of Wash Buffer (20X) and dilute to a final volume of 1200 mL with distilled or deionized water. Mix thoroughly. If a smaller volume of Wash Buffer (1X) is desired, add 1 volume of Wash Buffer (20X) to 19 volumes of distilled or deionized water. Wash Buffer (1X) is stable for 1 month at 2-8°C. Mix well before use.

2. **Substrate Solution:** Substrate A and Substrate B should be mixed together in equal volumes up to 15 minutes before use. Refer to the table below for correct amounts of Substrate Solution to prepare.

Strips Used	Substrate A (mL)	Substrate B (mL)	Substrate Solution (mL)
2 strips (16 wells)	1.5	1.5	3.0
4 strips (32 wells)	3.0	3.0	6.0
6 strips (48 wells)	4.0	4.0	8.0
8 strips (64 wells)	5.0	5.0	10.0
10 strips (80 wells)	6.0	6.0	12.0
12 strips (96 wells)	7.0	7.0	14.0

3. **IL-5 Standard:**

a) Two vials of Standards are provided in this kit to allow both serum/plasma and cell culture medium testing. Reconstitute the IL-5 Standard with either 2.0 mL of Calibrator Diluent I (for serum/plasma testing) or Calibrator Diluent II (for cell culture medium testing). This reconstitution produces a stock solution of 1000 pg/mL. Allow solution to sit for at least 15 minutes with gentle agitation prior to making dilutions. Use within one hour of reconstituting. The IL-5 standard stock solution can be stored frozen (-20°C) for up to 30 days. Avoid freeze-thaw cycles; aliquot if repeated use is expected.

b) Use the above stock solution to produce a serial 2-fold dilution series within the range of this assay (31.25 pg/mL to 1000 pg/mL) as illustrated. Add 0.5 mL of the appropriate Calibrator Diluent to each test tube. Between each test tube transfer be sure to mix contents thoroughly. The undiluted IL-5 Standard will serve as the **high standard (1000 pg/mL**) and the Calibrator Diluent will serve as the **zero standard (0 pg/mL).**

		0.5mL	0.5mL	0.5mL	0.5mL

0.5 mL
IL-5 Std

1000pg/mL	500pg/mL	250pg/mL	125pg/mL	62.5pg/ml	31.25pg/mL

ASSAY PROCEDURE

1. Prepare Wash Buffer (1X) and IL-5 Standards before starting assay procedure (see Preparation of Reagents). *It is recommended that the table and diagram provided be used as a reference for adding Standards or Samples to the Microtiter Plate.*

Wells	Contents		Wells	Contents	
1A, 1B	Standard 1 – 0 **pg/mL**	(S1)	**2C, 2D**	Standard 1 – 500 **pg/mL**	(S6)
1C, 1D	Standard 2– 31.25 **pg/mL**	(S2)	**2E, 2F**	Standard 1 – 1000 **pg/mL**	(S7)
1E, 1F	Standard 3– 62.5 **pg/mL**	(S3)	**2G-12H**	**IL-5** samples	
1G, 1H	Standard 4– 125 **pg/mL**	(S4)			
2A, 2B	Standard 5– 250 **pg/mL**	(S5)			

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S5	2	6	10	14	18	22	26	30	34	38
B	S1	S5	2	6	10	14	18	22	26	30	34	38
C	S2	S6	3	7	11	15	19	23	27	31	35	39
D	S2	S6	3	7	11	15	19	23	27	31	35	39
E	S3	S7	4	8	12	16	20	24	28	32	36	40
F	S3	S7	4	8	12	16	20	24	28	32	36	40
G	S4	1	5	9	13	17	21	25	29	33	37	41
H	S4	1	5	9	13	17	21	25	29	33	37	41

2. Add 50 μL of Standard or Sample to the appropriate well of the antibody pre-coated Microtiter Plate. Cover and incubate for 1 hour at room temperature.

3. Without washing the plate, add 50 μL Anti-IL-5 Biotin conjugate to each well. Mix well. Cover and incubate for 1 hour at room temperature.

4. Wash the Microtiter Plate using one of the specified methods indicated below:

   Manual Washing: Remove incubation mixture by aspirating contents of the plate into a sink or proper waste container. Using a squirt bottle, fill each well completely with Wash Buffer (1X) then aspirate contents of the plate into a sink or proper waste container. Repeat this procedure four more times for a **total of FIVE washes**. After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. *Note*: Hold the sides of the plate frame firmly when washing the plate to assure that all strips remain securely in frame.

   Automated Washing: Aspirate all wells, then wash plates **FIVE times** using Wash Buffer (1X). Always adjust your washer to aspirate as much liquid as possible and set fill volume at 350 μL/well/wash (range: 350-400 μL). After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. *It is recommended that the washer be set for a soaking time of 10 seconds or shaking time of 5 seconds between washes.*

5. Dispense 100 μl of Avidin Conjugate to each well. Cover and incubate for 1 hour at room temperature.

6. Prepare Substrate Solution no more than 15 minutes before end of second incubation (see Preparation of Reagents).
7. Repeat wash procedure as described in Step 4.

8. Add 100 μL Substrate Solution to each well. Cover and incubate for 15 minutes at room temperature.

9. Add 100 μL Stop Solution to each well. Mix well.

10. Read the Optical Density (O.D.) at 450 nm using a microtiter plate reader set within 30 minutes.

CALCULATION OF RESULTS

The standard curve is used to determine the amount of IL-5 in an unknown sample. The standard curve is generated by plotting the average O.D. (450 nm) obtained for each of the standard concentrations on the vertical (Y) axis versus the corresponding IL-5 concentration (pg/mL) on the horizontal (X) axis.

1.   First, calculate the mean O.D value for each standard and sample. All O.D. values are subtracted by the value of the zero-standard (0 pg/mL) or (S1) before result interpretation. Construct the standard curve using graph paper or statistical software.

2.   To determine the amount of IL-5 in each sample, first locate the O.D. value on the Y-axis and extend a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the corresponding IL-5 concentration., If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay. The concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

Results of a typical standard run of a IL-5 ELISA are shown below. Any variation in standard diluent, operator, pipetting and washing technique, incubation time or temperature, and kit age can cause variation in result. The following examples are for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain their own standard curve

<u>EXAMPLE ONE</u>

The following data was obtained for a standard curve using Calibrator Diluent I.

Standard (pg/mL)	O.D. (450 nm)	Mean	Zero Standard Subtracted (Std.) – (S1)
0	0.099, 0.101	0.100	0
31.25	0.164, 0.193	0.170	0.070
62.5	0.227, 0.301	0.237	0.137
125	0.393, 0.486	0.403	0.303
250	0.650, 0.849	0.685	0.585
500	1.118, 1.484	1.184	1.084
1000	2.105, 2.305	2.205	2.105
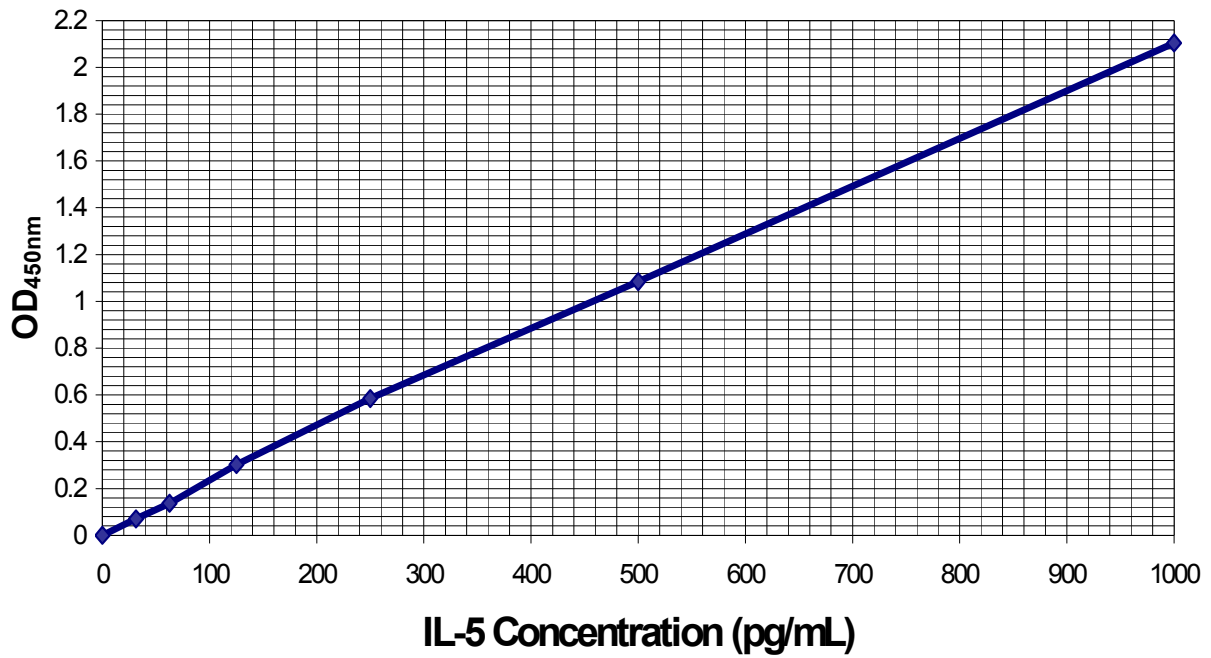
EXAMPLE TWO

The following data was obtained for a standard curve using Calibrator Diluent II.

Standard (pg/mL)	O.D. (450 nm)	Mean	Zero Standard Subtracted (Std.) – (S1)
0	0.049, 0.042	0.046	0
31.25	0.158, 0.160	0.159	0.113
62.5	0.202, 0.205	0.204	0.158
125	0.344, 0.346	0.345	0.299
250	0.585, 0.581	0.583	0.537
500	1.004, 1.072	1.038	0.992
1000	1.860, 1.857	1.715	1.669
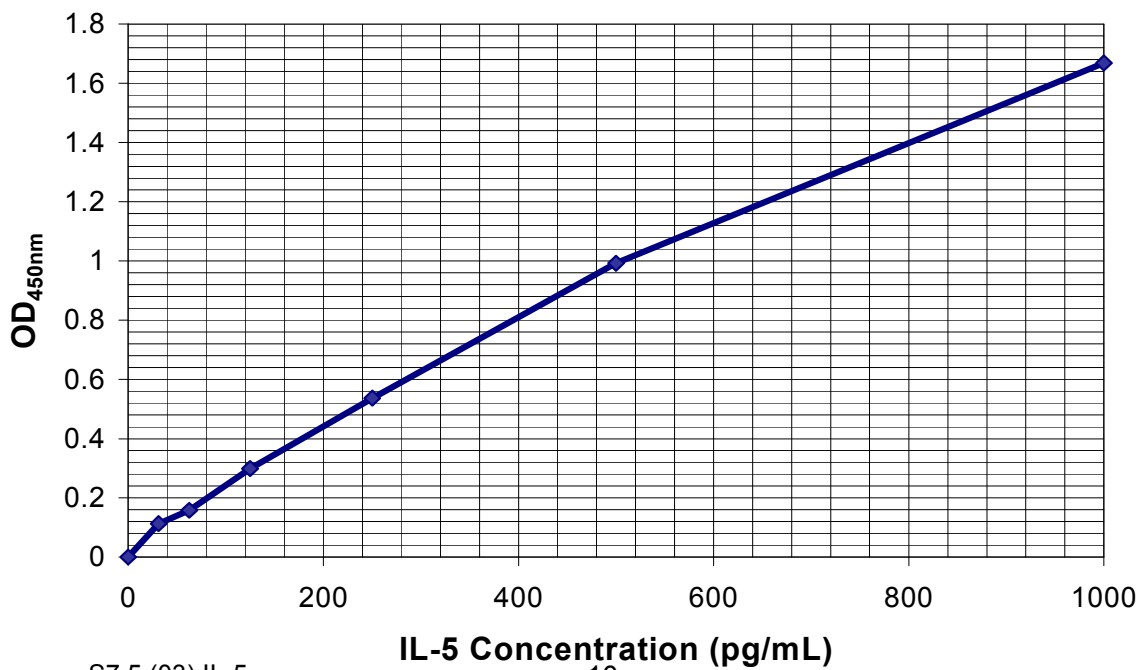
PERFORMANCE CHARACTERISTICS

1. INTRA-ASSAY PRECISION
To determine within-run precision, three different samples of known concentration were assayed by replicates of 20 in 1 assay.

	Calibrator Diluent I assay			Calibrator Diluent II assay		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	186	421	893	179	430	880
Standard Deviation (pg/mL)	9.1	32.5	51.0	8.7	35.5	42.1
Coefficient of Variation (%)	4.9	7.7	5.7	4.9	8.2	4.8

2. INTER-ASSAY PRECISION
To determine between-run precision, three different samples of known concentration were assayed by replicates on 20 different assays.

	Calibrator Diluent I assay			Calibrator Diluent II assay		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	193	442	886	180	428	901
Standard Deviation (pg/mL)	8.5	29.6	48.7	7.5	30.9	46.4
Coefficient of Variation (%)	4.4	6.7	5.5	4.2	7.2	5.1

3. RECOVERY
By employing various samples, the recovery of IL-5 was evaluated in 3 different amounts of IL-5 throughout the range of the assay.

Sample Type	**Average Recovery %**	**Range %**
Cell culture media	96	89 -108
Serum	94	83 - 101
Plasma	102	90 - 114

4. SENSITIVITY
The minimum detectable quantities of human IL-5 as observed by the standard curve generated for both Calibrator Diluent I and Calibrator Diluent II are 7.5 pg/mL and 6.3 pg/mL respectively. The two standard deviations above the mean optical density of the 20 replicates of the zero standard were defined as the minimum detectable quantities.

5. SPECIFICITY
This sandwich ELISA can detect both natural and recombinant human IL-5. This kit exhibits no detectable cross-reactivity with human; GM-CSF, GRO a, GRO b, INF-g, INF a, IL-1a, IL-1b, IL-1 RA, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, TNF-a, mouse IL-5 and IL-9.

6. **CALIBRATION**

This immunoassay is calibrated against WHO Standard (First International Standard, 1994. Code No.: 90/586).

7. **EXPECTED NORMAL VALUES**

Biological samples from apparently healthy, normal individuals were collected and the average IL-5 concentration was measured. All the measured samples of IL-5 concentrations were lower that the lowest standard (31.25 pg/ml).

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