Introduction and Background

A. Overview

Interleukin-16 (IL-16) discovered in 19821, known formerly as lymphocyte chemoattractant factor (LCF) is a lymphocyte chemoattractant factor of T cell origin with selective activity for CD4+ T cells. LCF was designated interleukin-16 (IL-16) in 1995. IL-16 is a product of CD8+ cells, CD4+ cells2, eosinophils3, mast cells and epithelial cells derived from asthmatics4. Expression of IL-16 in asthmatic epithelium correlates with the number of infiltrating CD4+ T cells5.

LCF was originally identified and purified from the supernatants of ConA-stimulated peripheral blood mononuclear cells (PBMC). In addition to its chemotactic activity, it is a competence growth factor selective for CD4+ T cells dependent on an interaction with CD4 for induction of functional activity. Its cDNA codes for a novel 14-kDa protein into a homotetrameric form is required for induction of biologic activity. IL-16 appears in culture supernatants as a relative molecular mass (Mr) ~56,000 biologically active, non-covalently linked tetramer, but migrates in monomeric form in SDS PAGE. Eluted monomeric peptides are inactive but reaggregate to Mr 56,000 regaining biological activity6. The protein expressed from the IL-16 cDNA demonstrates all the functions and chemical features of the native protein, including an identical pI, and autoaggregation into functional tetramers.

IL-16 is performed and stored in biologically active form in CD8+. T cells from which it is secreted following stimulation with histamine via H2 type receptors. The secretory process occurs within 4 hrs and does not require transcription, translation, or new protein synthesis.

IL-16's ability to inhibit the MLR and other antigen induced activation suggests that it may be useful in inhibiting allograft rejection. Because IL-16 selectively induces IL-2 responsiveness in CD4+ T cells it may be useful (along with IL-2) for selective CD4+ T cell immune reconstitution in individuals with lymphopenia, for example following chemotherapy or HIV-1 infection. In the latter circumstance, its ability to inhibit HIV transcription should provide a protective role if used as a therapy in HIV-1 infected individuals7.

Inhibitors of IL-16 chemotactic function may be useful in diseases in which it appears to play a prominent role in the inflammatory process. The presence of IL-16 early after antigen challenge in asthma8, the marked upregulation of IL-16 synthesis by epithelium of asthmatics along with the correlation of IL-16 protein with the number of infiltrating CD4+ T cells in the airways of human asthmatics suggest that therapeutics aims at blocking IL-16 synthesis or function may be valuable in this disease.

This IL-16 ELISA is a 4.5 hour solid phase immunoassay readily applicable to measure IL-16 in serum, plasma, cell culture supernatant, and other biological fluids in the range of 0 to 3200 pg/mL. It showed no cross-reactivity with other cytokines tested such as EGF, EPO, GM-CSF, IL-1β, IL-7, IL-8, IFN-γ, MCAF, MCP-3, M-CSF, SAA, TGF-β, and TNF-α.

B. Test Principle

This IL-16 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 for IL-16. Standards or samples are then added to the appropriate microtiter plate wells and incubated. IL-16 if present, will bind and become immobilized by the antibody pre-coated on the wells. The microtiter plate wells are thoroughly washed to remove any unbound IL-16 and other components of sample. In order to quantitatively determine the amount of IL-16 present in the sample, a standardized preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody specific for IL-16 is added to each well to "sandwich" the IL-16 immobilized during the first incubation. The microtiter plate then undergoes a second incubation. The wells are thoroughly washed to remove all unbound HRP-conjugated antibodies and a TMB (3,3'5,5' tetramethyl-benzidine) substrate solution is added to each well. The enzyme (HRP) and substrate solution are allowed to react over a short incubation period. Only those wells that contain IL-16 and enzyme-substrate reaction will exhibit a change in colour. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the colour change measured spectrophotometrically at a wavelength of 450 nm ± 2 nm.

In order to measure the concentration of IL-16 in the samples, this kit includes two calibration diluents (Calibrator Diluent I for serum/plasma testing and Calibrator Diluent II for cell culture supernatant testing). According to the testing system, the provided standard is diluted (2-fold) with the appropriate Calibrator Diluent and assayed at the same time as the samples. This allows the operator to produce a standard curve of Optical Density (O.D) versus IL-16 (pg/mL). The concentration of IL-16 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

C. Notice for Application of Kit

- ✓ This kit has been configured for research use only and is not for diagnostic and clinical use.
- ✓ 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.
- ✓ Allow kit reagents and materials to reach room temperature (20-25℃) before use. Do not use water baths to thaw samples or reagents.
- ✓ Do not use kit components beyond their expiration date.
- ✓ Use only deionized or distilled water to dilute reagents.
- ✓ Do not remove microtiter plate from the storage bag until needed. Unused strips should be stored at 2-8℃ in their pouch with the desiccant provided.
- ✓ Use fresh disposable pipette tips for each transfer to avoid contamination.
- ✓ 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.

- ✓ All samples should be disposed of in a manner that will inactivate human viruses.

  Solid Wastes: Autoclave for 60 minutes at 121℃.

  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.

- ✓ Substrate Solution is easily contaminated. If bluish prior to use, do not use.
- ✓ Substrate B contains 20% acetone, keep this reagent away from sources of heat or flame.

Material and Method

A. List of component

1. One 96-well Mcrotiter Pate precoated with anti-human IL-16 monoclonal antibody.
2. IL-16 Conjugate (Anti-human IL-16 polyclonal antibody conjugated to horseradish peroxidase with preservative): 25ml.
3. hGH Standard, 12.8 ng/vial (Recombinant human IL-16 in a buffered protein base with preservative, lyophilized): 2 vials.
4. Calibrator Diluent I (Animal serum with preservative. For serum/plasma testing): 30 ml.
5. Calibrator Diluent II (Cell culture medium with calf serum and preservative. For cell culture supernatant testing): 50 ml.
6. Wash Buffer (20X) (20-fold concentrated solution of buffered surfactant): 60ml.
7. Substrate A (Buffered solution with H_2O_2): 16ml.
8. Substrate B (Buffered solution with TMB): 16ml.
9. Stop Solution (2N Sulphuric Acid (H_2SO_4). Caution: Caustic Material!): 14ml.

B. Additional Required Materials But Not Provided

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. Incubator (37℃).
7. Microtiter plate reader (450 nm ± 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.

C. Sample preparation

Collection, Handling, and Storage

a. **Cell Culture Supernatant:** 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℃) 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℃) to remove any particulate. This IL-16 ELISA kit is not affected by haemolysis of specimens. No adverse effects have been noted in the presence of anti-coagulants, sodium citrate, EDTA, or heparin.

- Avoid grossly hemolytic, lipidic or turbid samples.
- Serum, plasma, and cell culture supernatant samples to be used within 24-48 hours may be stored at 2-8℃, otherwise samples must stored at -20℃ 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.

Dilution Procedures

a. Serum/plasma samples: No dilution required. Directly add 200 µL of sample to each well

b. Cell culture supernatant samples with 5-20% animal serum: No dilution required. Directly add 200 µL of sample to each well.

c. Urine, water based, or serum free cell culture supernatant samples: Use Calibrator Diluent II to make a 1:1 dilution. Directly add 100 µL of sample to each well, then add 100 µL of Calibrator Diluent II.

Reagent Preparation and Storage

Remove all kit reagents from refrigerator and allow them to reach room temperature (20-25℃). 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℃. 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)	3.0	3.0	6.0
4 strips (32 wells)	6.0	6.0	12.0
6 strips (48 wells)	8.0	8.0	16.0
8 strips (64 wells)	10.0	10.0	20.0
10 strips (80 wells)	12.0	12.0	24.0

12 strips (96 wells)	14.0	14.0	28.0

3. IL-16 Standard:

a. Two vials of Standard are provided in this kit to allow both serum/plasma and cell culture supernatant testing. Reconstitute IL-16 Standard with either 4.0 mL of Calibrator Diluent I (for serum/plasma testing) or Calibrator Diluent II (for cell culture supernatant testing). This reconstitution produces a stock solution of 3200 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-16 standard stock solution can be stored frozen (-20℃) 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 as illustrated below. Add 1.0 mL of the appropriate Calibrator Diluent to each test tube. Between each test tube transfer be sure to mix contents thoroughly. The undiluted 1L-16 Standard will serve as the high standard (3200 pg/mL) and the Calibrator Diluent will serve as the zero standard (0 pg/mL).

D. Stability and storage

All reagents provided are stored at 4℃. Refer to the expiration date on the label.

E. Protocol

1. Prepare Wash Buffer and IL-16 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 and Samples to the Microtiter Plate.

Wells	Contents	Wells	Contents
1A, 1B	Standard 1 - **0 pg/mL** (S1)	**2A, 2B**	Standard 5 - **800 pg/mL** (S5)
1C, 1D	Standard 2 - **100 pg/mL** (S2)	**2C, 2D**	Standard 6 - **1600 pg/mL** (S6)
1E, 1F	Standard 3 - **200 pg/mL** (S3)	**2E, 2F**	Standard 7 - **3200 pg/mL** (S7)
1G, 1H	Standard 4 - **400 pg/mL** (S4)	**2G-12H**	samples

	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 200 µL of Standard or Sample (Urine, water based, or serum free cell culture supernatant samples

need to dilute 1:1--see sample preparation, pg 6) to the appropriate well of the antibody pre-coated wells of the Microtiter Plate. Mix well. Cover and incubate for 2 hours at room temperature.

3. 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.
4. Add 200 µL of conjugate to each well. Cover and incubate for 2 hours at room temperature.
5. Prepare Substrate Solution (see Preparation of Reagents) no more than 15 minutes before end of second incubation.
6. Repeat wash procedure as described in Step 3.
7. Add 200 µL Substrate Solution to each well. Cover and incubate for 15 minutes at room temperature.
8. Add 100 µL Stop Solution to each well. Mix well.
9. Read the Optical Density (O.D.) at 450 nm using a microtiter plate reader within 30 minutes.

F. Calculation of results

The standard curve is used to determine the amount of IL-16 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-16 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) before result interpretation. Construct the standard curve using graph paper or statistical software.
2. To determine the amount of IL-16 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-16 concentration. If samples have been diluted (Urine, water based, or serum free cell culture supernatant samples), the concentration read from the standard curve must be multiplied by the dilution factor (× 2).
3. If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay. Dilution factor must be applied to calculation of results.

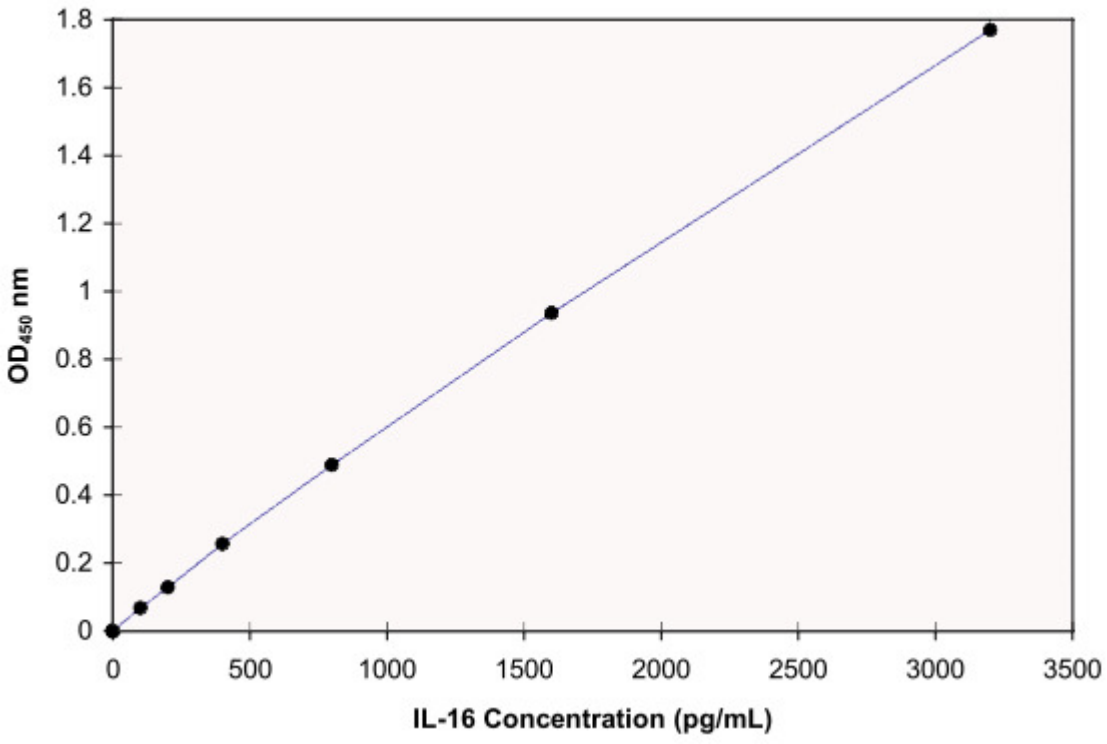
G. Typical Data

Results of a typical standard run of a IL-16 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 his or her own standard curve

Example One

Results of a typical standard run are shown below:

Standard (pg/mL)	O.D. (450 nm)	Mean	Zero Standard Subtracted (Std.) - (S1)
0	0.044, 0.046	0.045	0
100	0.089, 0.091	0.090	0.045
200	0.160, 0.158	0.159	0.114
400	0.258, 0.260	0.259	0.214
800	0.470, 0.472	0.471	0.426
1600	0.890, 0.900	0.895	0.850
3200	1.702, 1.710	1.706	1.661



H. Performance Characteristics

1. **Intra-assay Precision**

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

References

1. Iranmesh A, et al. J Clin Endocrinol Metab 73:1081-1088, 1991.
2. Smal J, et al. Biochem Biophys Res Comm 134:159-165, 1986.
3. Frasier SD. Endocrin Rev 4:155-170, 1983.
4. Ad Hoc Committee on Growth Hormone Usage, et al. Pediatrics 72:891894, 1983.