Human IL-12 (P70) ELISA Kit

For the quantitative determination of human interleukin 12 (IL-12) (P70) concentrations in serum, plasma, cell culture supernatant, and other biological fluids

Catalogue Number: EL10032

96 tests

FOR LABORATORY RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

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S7.5 (03) IL-12

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INTENDED USE

This Human Interleukin 12 (P70) ELISA Kit is to be used for the *in vitro* quantitative determination of human interleukin 12 (IL-12) concentrations in serum, plasma, cell culture supernatant, and other biological fluids. This kit is FOR LABORATORY RESEARCH USE ONLY and is not for use in diagnostic or therapeutic procedures.

INTRODUCTION

Interleukin 12 (IL-12), also known as natural killer cell stimulatory factor (NKSF) and cytotoxic lymphocyte maturation factor (CLMF), is a pleitopic cytokine originally identified in the medium of cultured EBV-transformed RPMI-8866 cells) (1-3). IL-12 is a 75kDa glycoprotein heterodimer composed of two unequal, genetically-unrelated subunits. The smaller subunit (p35) has homology to IL-6 and G-CSF while the larger subunit (P40) shows recognizable similarity to the soluble receptor for IL-6, leading to the suggestion that IL-12 might have evolved from a cytokine/soluble receptor complex (2-6). Cells known to produce IL-12 include monocytes/macrophages, B cells and connective tissue type mast cells (7-10). IL-12 shows species specificity with human IL-12 reportedly showing minimal activity in the murine system (3,7). For reviews on IL-12, see references (8-12).

Each subunit of IL-12 apparently arises from a single copy gene. The transcription of the subunit of the subunit mRNAs is closely co-ordinated, although an excess of the larger subunit (P40) has been shown to be produced by B cells in addition to active IL-12 (1,3). Expression of the smaller chain (p35) is reported to be enhanced by simultaneous expression of the larger chain (p40). Although IL-12 activity cannot be demonstrated in the absence of either chain, the presence of only p40 is associated with inhibition of IL-12-associated activities (3,4). As suggested by their names, p35 has a native molecular weight of 35 kDa while p40 has a native molecular weight of 40 kDa. In humans, p35 is 197 amino acid residues in length with a predicted molecular weight of 22.5 kDa. The molecule contains 7 cysteine residues plus 3 potential N-glycosylation sites and the molecule is believed to be heavily glycosylated. The p40 subunit is 306 amino acid residues in length with a predicted molecular weight of 34.7 kDa. The molecule contains 10 cysteine residues and four potential N-linked glycosylation sites (3). The murine p35 subunit shows 60% sequence identity with the corresponding human subunit and is 193 amino acid residues in length with seven conserved cysteines and one possible N-linked glycosylation site. Murine p40 shows 70% sequence identity to human p40 and is 313 amino acid residues in length with eleven conserved cysteines and three potential N-linked glycosylation sites (7). In both human and mouse p35 and p40, the mature molecules separate functions can be attributed to p35 and p40. It is not clear what separate functions can be attributed to p35 and p40. Preliminary evidence suggests however that p40 is involved in receptor binding and p35 is important for signal transduction (13).

A unique high affinity receptor for IL-12 (IL-12R) has been characterized from PHA-stimulated human peripheral blood mononuclear cells (14). Approximately 110 kDa as determined by cross-linking studies, it has a K_d in the range of 100-600 pM (14). Cross-linking studies also suggested an association with a second protein of approximately 85 kDa. IL-12 receptor has also been reported to be present on PHA or IL-2 stimulated CD4+, CD8+, and CD56+ cells and on one T cell and one NK cell line (14, 15).

IL-12 is produced by macrophages and B lymphocytes and has been shown to have multiple effects on T cells and natural killer (NK) cells (16, 17). These include inducing production of IFN-γ and TNF by resting and activated T and NK cells, synergizing with other IFN-γ inducers at both the transcriptional and post-transcriptional levels to induce IFN-γ gene expression, enhancing the cytotoxic activity of resting NK an d T cells, inducing and synergizing with IL-2 in the generation of lymphokine-activated killer (LAK) cells, acting as a comitogen to stimulate proliferation of resting T cells and inducing proliferation of activated T and NK cells (16). Evidence indicates that IL-12 produced by macrophages in response to infectious agent, is a central mediator of the cell-mediated immune response by its actions on the development, proliferation, and activities of TH1 cells (8,9, 18,19). In its role as the initiator of cell-mediated immunity, it has been suggested that IL-12 has therapeutic potential as a stimulator of cell-mediated immune responses to microbial pathogens, metastatic cancer, and viral infections such as AIDS (8,9, 18-20).

PRINCIPLE OF THE ASSAY

This IL-12 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-12. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated monoclonal antibody preparation specific for IL-12 and incubated. IL-12 if present, will bind and become immobilized by the antibody pre-coated on the wells and then be “sandwiched” by biotin conjugate. The microtiter plate wells are thoroughly washed to remove unbound IL-12 and other components of the sample. In order to quantitatively determine the amount of IL-12 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 that each have 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-12, 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.

In order to measure the concentration of IL-12 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-12 concentration (pg/mL). The concentration of IL-12 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

LIMITATIONS OF THE PROCEDURE

- FOR LABORATORY RESEARCH USE ONLY, NOT FOR USE IN DIAGNOSTIC PROCEDURES.

- As manufacturers we take great care to ensure that our products are suitable for use with all validated sample types, as designated in the product insert. However, it is possible that in some cases, high levels of interfering factors may cause unusual results.

- The kit should not be used beyond the expiration date on the kit label.

- It is important that the Calibrator Diluent selected for the standard curve be consistent with the samples being assayed.

- If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay.

- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

- Soluble receptors or other binding proteins present in biological samples do not necessarily interfere with the measurement of ligands in samples. However, until the factors have been tested, the possibility of interference cannot be excluded.

REAGENTS PROVIDED

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

	96 wells

1. **IL-12 MICROTITER PLATE** (EL32-1) ***96 wells***
   Pre-coated with anti-human IL-12 monoclonal antibody.

2. **BIOTIN CONJUGATE** (EL32-2) ***6 mL***
   Anti-human IL-12 monoclonal antibody conjugated to Biotin.

3. **AVIDIN CONJUGATE** (EL32-3) ***12 mL***
   Avidin conjugated to horseradish peroxidase.

4. **IL-12 STANDARD** (EL32-4) ***2 vials***
   Recombinant human IL-12 (2000 pg/vial) in a buffered protein base with preservative, lyophilized.

5. **CALIBRATOR DILUENT I** (EL32-5) ***25 mL***
   Animal serum with buffer and preservative. *For serum/plasma testing.*

6. **CALIBRATOR DILUENT II** (EL32-6) ***25 mL***
   Cell culture medium with calf serum and preservative. *For cell culture supernatant testing.*

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

8. **SUBSTRATE A** (EL32-7) ***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 ± 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 Waste: Autoclave 60 min. at 121°C.
   Liquid Waste: 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 viruses 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 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°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, and cell culture supernatant 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.

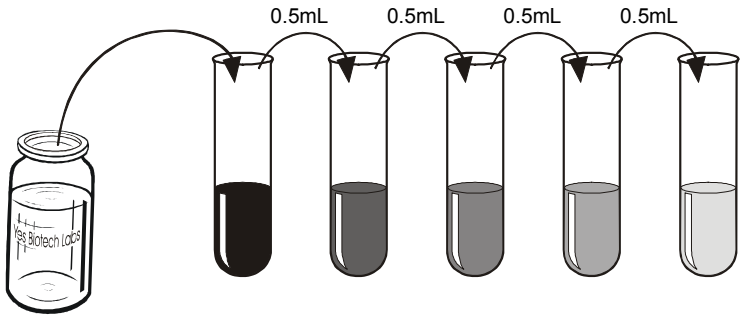
2. **Substrate Solution:** Substrate A and Substrate B should be mixed together in equal volumes up to 15 minutes before use. Refer to the table provided for correct amounts of Substrate Solution to prepare.

Strips **Used**	**Substrate A (mL)**	**Substrate B (mL)**	**Substrate Solution (mL)**
2 strips (16 wells)	2.0	2.0	4.0
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-12 Standard:**

a) Two vials of Standard are provided in this kit to allow both serum/plasma and cell culture supernatent testing. Reconstitute IL-12 Standard with 2.0 mL of Calibrator Diluent I or Calibrator Diluent II. 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-12 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, as described below, within the range of this assay (31.25 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-12 stock solution will serve as the high standard **(**1000 pg/mL) and the Calibrator Diluent will serve as the zero standard (0 pg/mL).



IL-12 Standard 1,000 pg/ml	500 pg/ml	250 pg/ml	125pg/ml	62.5 pg/ml	31.25 pg/ml

ASSAY PROCEDURE

1. Prepare Wash Buffer and IL-12 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	**250 pg/mL**	(S5)
1C, 1D	Standard 2	**31.25 pg/mL**	(S2)	**2C, 2D**	Standard 6	**500 pg/mL**	(S6)
1E, 1F	Standard 3	**62.5 pg/mL**	(S3)	**2E, 2F**	Standard 7	**1000 pg/mL**	(S7)
1G, 1H	Standard4	**125 pg/mL**	(S4)	**2G,2H**	**IL-12** 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 100μL of Standard or Sample to the appropriate well of the antibody pre-coated Microtiter Plate and incubate 1 hour at room temperature.

3. Without discarding the standards and samples, add 50μL IL-12 Biotin conjugate to each wells. 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 100µl of Avidin Conjugate to each well Mix 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 100µL Substrate Solution to each well. Cover and incubate for 15 minutes at room temperature.

9. Add 100µL 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-12 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-12 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-12 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-12 concentration. If samples generate values higher than the highest standard, dilute the samples and repeat the assay, the concentration read from the standard curve must be multiplied by the dilution factor.

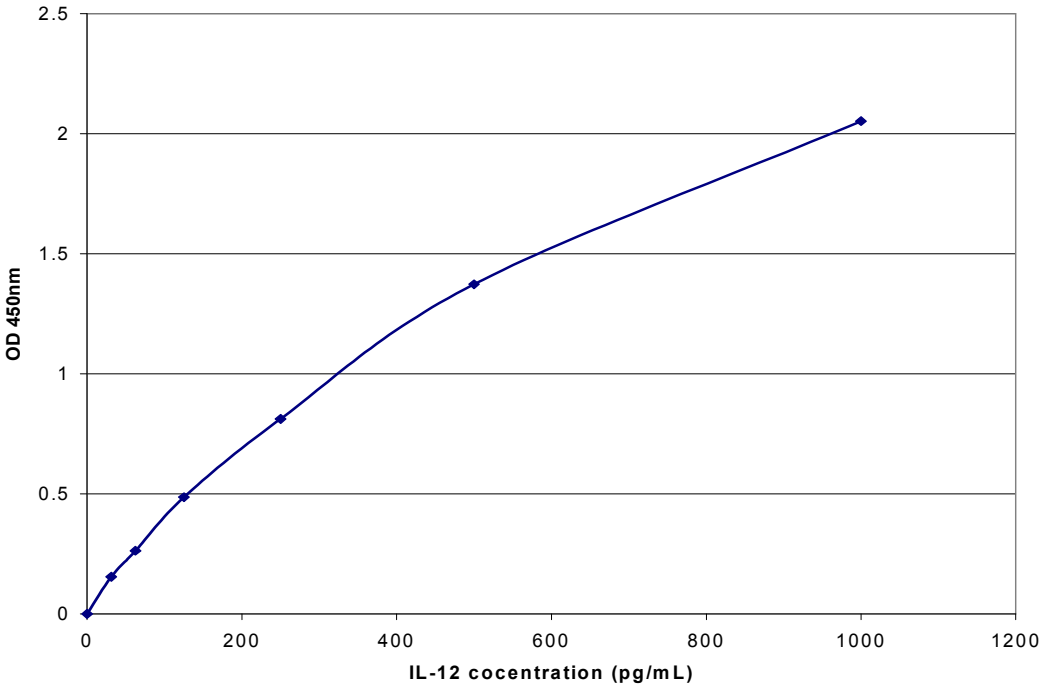
TYPICAL DATA

Results of a typical standard run of an IL-12 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.

EXAMPLE ONE

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

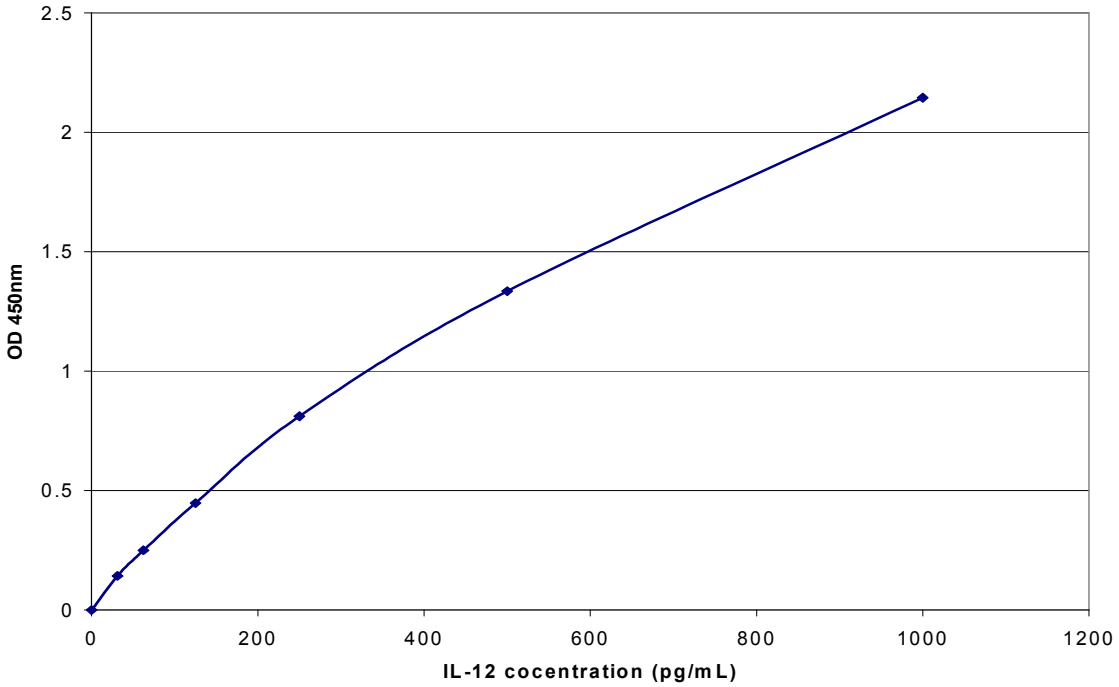
Standard (pg/mL)	**O.D. (450 nm)**	**Mean**	**Zero Standard Subtracted** (Std.) - (S1)
0	0.038, 0.032	0.035	0
31.25	0.190, 0.190	0.190	0.155
62.5	0.294, 0.310	0.298	0.263
125	0.506, 0.533	0.520	0.485
250	0.866, 0.829	0.847	0.812
500	1.395, 1.429	1.408	1.373
1000	2.187, 1.992	2.102	2.052



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	0.042, 0.064	0.053	0
31.25	0.186, 0.194	0.190	0.143
62.5	0.292, 0.314	0.303	0.250
125	0.508, 0.494	0.501	0.448
250	0.883, 0.846	0.864	0.811
500	1.382, 1.393	1.388	1.335
1000	2.155, 2.242	2.198	2.145



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.

	Calibrator Diluent I Assay		
Sample	**1**	**2**	**3**
n	20	20	20
Mean (pg/mL)	31.2	456	896
Standard Deviation	3.5	14.7	9.7
Coefficient of Variation (%)	11.2	3.3	1.1

	Calibrator Diluent II Assay		
Sample	**1**	**2**	**3**
n	20	20	20
Mean (pg/mL)	31.2	401	905
Standard Deviation	3.5	18.6	17.8
Coefficient of Variation (%)	11.2	4.5	2.0

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		
Sample	**1**	**2**	**3**
n	6	6	6
Mean (pg/mL)	35.1	432	902
Standard Deviation	2.8	27	28.8
Coefficient of Variation (%)	7.9	6.5	3.3

	Calibrator Diluent II Assay		
Sample	**1**	**2**	**3**
n	6	6	6
Mean (pg/mL)	30.3	479	925
Standard Deviation	2.9	45.1	10.2
Coefficient of Variation (%)	9.6	0.9	1.1

3. **RECOVERY**

The recovery of IL-12 spiked to seven different levels in five test samples throughout the range of the assay was evaluated. All samples were mixed and assayed in duplicate.

Sample Type	***Average Recovery (%)***	***Range (%)***
Cell Culture Media	98	82-97
Serum	84	72-91
EDTA plasma	81	82-90
Heparin plasma	70	60-84
Citrate plasma	100	85-101

4. **SENSITIVITY**

The minimum detectable dose of IL-12 was determined by adding two standard deviations to the mean optical density value of 20 zero standard replicates and calculating the corresponding concentration from the standard curve. The minimum detectable dose of human IL-12 using a standard curve generated with Calibrator Diluent I is <5.0 pg/mL and using Calibrator Diluent II is <5.1 pg/mL.

5. **SPECIFICITY**

This sandwich ELISA recognises both natural and recombinant human IL-12. This kit exhibits no significant cross-reactivity with human IL-1α, IL-β IL-2, IL-3, IL-4, IL-5 IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12 p40 IL-13 IL-15, GRO α GRO β, IFN γ or mouse IL-12 p70 and IL-12 p40.

6. **CALIBRATION**
   This immunoassay is calibrated against natural human IL-12. (NIBSC/WHO First International Standard 95/544).

7. **EXPECTED NORMAL VALUES**
   Biological samples from apparently healthy, normal individuals were collected and the average IL-12 concentration measured. Serum samples (n=15) average value: 85 pg/mL, range: 35-175pg/mL whereas plasma samples (n=16) average value: 97 pg/mL, range: 35-212 pg/mL.

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