ELISA Kit

Catalog #KAC1568

Human

IL-12 p70

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PURPOSE

The Invitrogen Human Interleukin-12 (Hu IL-12 p70) ELISA is to be used for the quantitative determination of Hu IL-12 in human serum, plasma, buffered solution, or cell culture medium in the range of 1.56 to 100 pg/mL. The assay will exclusively recognize both natural and recombinant Hu IL–12 heterodimer.

INTRODUCTION

Human interleukin-12 (IL-12) is a 70 kDa (p70) lymphokine produced mainly by monocytes, macrophages, B-lymphocytes and dendritic cells. IL-12 shows an unusual heterodimeric structure composed of one 40 kDa (p40) and one 35 kDa (p35) subunit linked together by disulfide bonds. p35 subunit is distantly related to IL-6 and G-CSF while p40 shows homology to the extracellular domain of the α chain of the IL-6 receptor. This suggests that IL-12 may have evolved from a cytokine/ soluble receptor complex.

p40 is secreted in large excess over the biologically active heterodimer. p40 is involved in receptor binding, but p35 is necessary for signal transduction. Monomers and mainly homodimers of p40 show antagonist activity to IL-12.

In vivo, IL-12 appears to play a major role in auto-immune disease, in the resistance to bacterial and parasitic infections, in antiviral responses including HIV, and in the promotion of anti-tumor immunity. IL-12 has been shown to be a powerful adjuvant in vaccination.

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Read entire protocol before use.

PRINCIPLE OF THE METHOD

The Invitrogen Hu IL-12 p70 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for Hu IL-12 has been coated onto the wells of the microtiter strips provided. Samples, including standards of known Hu IL-12 content, control specimens, and unknowns, are pipetted into these wells, followed by the addition of a biotinylated second monoclonal antibody.

During the first incubation, the Hu IL-12 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 Hu IL-12 present in the original specimen.

REAGENTS PROVIDED

Note: *Store all reagents at 2 to 8°C.*

Reagent	***96 Test Kit***	***192 Test Kit***
Hu IL-12 p70 Standard, recombinant Hu IL-12. Refer to vial label for quantity and reconstitution volume.	2 vials	4 vials
Standard Diluent Buffer. Contains 8 mM sodium azide, 0.05% Proclin® 300, 0.05% thymol and 0.1% benzamidine; 25 mL per bottle.	1 bottle	2 bottles
Hu IL-12 p70 Antibody-Coated Wells, 96 wells per plate.	1 plate	2 plates
Incubation Buffer, contains 8 mM sodium azide; 11 mL per bottle.	1 bottle	1 bottle
Hu IL-12 p70 Biotin Conjugate, (Biotin-labeled anti-IL-12 p70). Contains 8 mM sodium azide; 6 mL per bottle.	1 bottle	2 bottles
Streptavidin-Peroxidase (HRP), (100x) concentrate. Contains 3.3 mM thymol; 0.125 mL per bottle.	1 vial	2 vials
Streptavidin-Peroxidase (HRP) Diluent. Contains 0.05% Proclin® 300; 25 mL per bottle.	1 bottle	1 bottle
Wash Buffer Concentrate (25x); 100 mL per bottle.	1 bottle	1 bottle
Stabilized Chromogen, Tetramethylbenzidine (TMB); 25 mL per bottle.	1 bottle	1 bottle
Stop Solution; 25 mL per bottle.	1 bottle	1 bottle
Plate Covers, adhesive strips.	3	4

Disposal Note: This kit contains materials with small quantities of sodium azide and Proclin® 300. 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.

SUPPLIES REQUIRED BUT 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. Distilled or deionized water.
4. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
5. Data analysis and graphing software. 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.

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 to 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 >100 pg/mL should be further 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. In-house 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.

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 of infectious agents.

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. High pressure jets of some automated washers may adversely affect reproducibility.

REAGENT PREPARATION AND STORAGE

A. Reconstitution and Dilution of Hu IL-12 p70 Standard

This assay has been calibrated against the WHO reference preparation (NIBSC, Hertfordshire, UK, EN6 3QG). One picogram of standard equals 10 mIU of NIBSC standard (95/544).

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 gently and allow to sit for 10 minutes to ensure complete reconstitution.
2. Add 0.020 mL of the reconstituted standard to a tube containing 0.980 mL *Standard Diluent Buffer*. Label as 100 pg/mL Hu IL-12. Mix.
3. Add 0.300 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 50, 25, 12.5, 6.25, 3.12 and 1.56 pg/mL Hu IL-12.
4. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution of Hu IL-12 p70

Standard:	Add:	Into:
100 pg/mL	Prepare as described in Step 2.	
50 pg/mL	0.300 mL of the 100 pg/mL std.	0.300 mL of the Diluent Buffer
25 pg/mL	0.300 mL of the 50 pg/mL std.	0.300 mL of the Diluent Buffer
12.5 pg/mL	0.300 mL of the 25 pg/mL std.	0.300 mL of the Diluent Buffer
6.25 pg/mL	0.300 mL of the 12.5 pg/mL std.	0.300 mL of the Diluent Buffer
3.12 pg/mL	0.300 mL of the 6.25 pg/mL std.	0.300 mL of the Diluent Buffer
1.56 pg/mL	0.300 mL of the 3.12 pg/mL std.	0.300 mL of the Diluent Buffer
0 pg/mL	0.300 mL of the Diluent Buffer	An empty tube

Discard all remaining reconstituted and diluted standards after completing assay. Return *Standard Diluent Buffer* to the refrigerator.

C. Storage and Final Dilution of Streptavidin-HRP

Please Note: The *Streptavidin-HRP* 100x concentrate is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow *Streptavidin-HRP* concentrate to reach room temperature. Gently mix. Pipette *Streptavidin-HRP* concentrate slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Dilute 10 µL of the 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.

D. 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.

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: **Please add the reagents in the following order. 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 50 µL of *Incubation Buffer* to the wells corresponding to standards and serum/plasma samples or 50 µL of Standard Diluent Buffer to the wells corresponding to buffered solutions or cell culture samples. Wells reserved for chromogen blank should be left empty.
3. Add 100 µL of *Standard Diluent Buffer* to zero wells. Well(s) reserved for chromogen blank should be left empty.

4. Add 100 µL of standards, samples or controls to the appropriate microtiter wells. (See **REAGENT PREPARATION AND STORAGE**, Section B.)
5. Pipette 50 µL of biotinylated anti-IL-12 p70 (*Biotin Conjugate*) solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.
6. Cover plate with *plate cover* and incubate for **3 hours at room temperature**.
7. Thoroughly aspirate or decant solution from wells and discard the liquid. Gently wash wells 4 times. See **DIRECTIONS FOR WASHING**.
8. 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.)
9. Cover plate with the *plate cover* and incubate for **30 minutes at room temperature**.
10. Thoroughly aspirate or decant solution from wells and discard the liquid. Gently wash wells 4 times. See **DIRECTIONS FOR WASHING**.
11. Add 100 µL of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.
12. 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 microtiter plate reader used often determines the incubation time for chromogen substrate. 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.

13. 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.
14. 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*.
15. 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.
16. Read the Hu IL-12 concentrations for unknown samples and controls from the standard curve plotted in step 15. (Samples producing signals greater than that of the highest standard (100 pg/mL) should be diluted in *Standard Diluent Buffer* for serum/plasma samples or corresponding medium for cell culture samples and reanalyzed, 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 100 pg/mL Hu IL-12.

Standard Hu IL-12 p70 (pg/mL)	Optical Density (450 nm)
0	0.115
	0.110
1.56	0.184
	0.190
3.12	0.280
	0.293
6.25	0.425
	0.438
12.5	0.642
	0.655
25	1.095
	1.159
50	1.880
	1.889
100	2.890
	2.874

LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 100 pg/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples >100 pg/mL with *Standard Diluent Buffer* or corresponding medium, according to the samples. Reanalyze 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 Hu IL-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, binding molecules and soluble receptors. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.

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PERFORMANCE CHARACTERISTICS

SENSITIVITY

The minimum detectable dose of Hu IL-12 is 0.2 pg/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 24 times.

PRECISION

1. Intra-Assay Precision

Samples of known Hu IL-12 concentration were assayed in replicates of 24 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	4.5	13.8	48.3
SD	0.34	0.89	3.82
%CV	7.6	6.4	7.9

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 16 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	3.8	11.9	44.4
SD	0.31	0.87	3.64
%CV	8.2	7.3	8.2

SD = Standard Deviation
CV = Coefficient of Variation

LINEARITY OF DILUTION

Human serum or cell culture samples containing Hu IL-12 were serially diluted over the range of the assay in *Standard Diluent Buffer* or *RPMI* containing 10% fetal calf serum, respectively. Linear regression analysis of samples versus the expected concentration yielded an average correlation coefficient of 0.99.

	Serum			Cell Culture			
Dil.	**Measured (pg/mL)**	**Expected (pg/mL)**	**% Expected**	**Dil.**	**Measured (pg/mL)**	**Expected (pg/mL)**	**% Expected**
1/1	72.7	-	-	1/1	64.6	-	-
1/2	35.5	36.3	98	1/2	34.7	32.3	107
1/4	18.1	18.2	100	1/4	15.8	16.2	98
1/8	8.8	9.1	97	1/8	8.4	8.1	104
1/16	4.9	4.5	108	1/16	3.8	4.0	94

RECOVERY

The recovery of Hu IL-12 added to human serum, human EDTA plasma, human heparinized plasma and human citrated plasma averaged 87%, 99%, 97% and 95%, respectively. The recovery of Hu IL-12 added to tissue culture medium containing both 1% and 10% fetal calf serum averaged 98% and 102%, respectively.

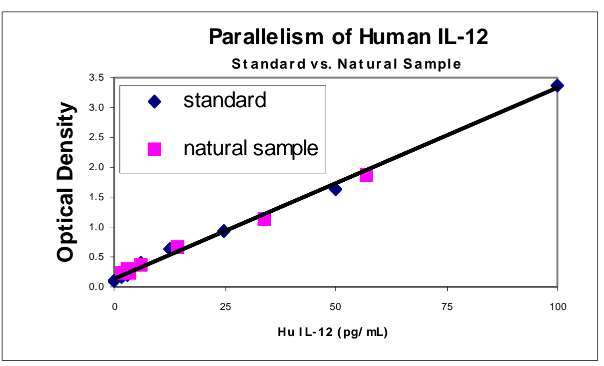
Note: Low recoveries were observed in some serum/plasma samples. Addition of Pefabloc at a final concentration of 1 mg/mL in these samples restored 90 to 100% recoveries. Pefabloc is a serine protease inhibitor supplied by Roche (Cat. # 11 429 868 001).

SPECIFICITY

Buffered solutions of a panel of substances at 150 ng/mL were assayed with the Invitrogen Hu IL-12 p70 kit. The following substances were tested and found to have no cross-reactivity: human IFN-γ, TNF-α, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-16, IP-10, GRO, MCAF, MCP3, MIP-1α, SCF, Oncostatin-M, LIF, Leptin. Human IL-23 and the p40 subunit of Hu IL-12 were found to have no significant cross-reactivity (<0.01%) and did not interfere with the quantitation of Hu IL-12 p70.

PARALLELISM

Natural Hu IL-12 was serially diluted in *Standard Diluent Buffer*. The optical density of each dilution was plotted against the standard curve. Parallelism between the natural and recombinant protein was demonstrated by the figure below and indicated that the standard accurately reflects natural Hu IL-12 content in samples.



EXPECTED VALUES

Serum/Plasma: forty serum and EDTA plasma samples were evaluated in the assay.
Detectable: > 0.2 pg/mL
ND : non detectable

Sample	N	Range (pg/mL)	% Detectable	Mean of Detectable (pg/mL)
Serum	20	ND—0.79	40	0.47
EDTA plasma	20	ND—0.62	45	0.47

Cell culture:
Human monocytic cells (THP-1) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate. Cells were pre-treated for 6 days with 75 ng/mL of GM-CSF, then stimulated with:

- 1 µg/mL of LPS
- 1 µg/mL of LPS + 1 µg/mL IFN-γ
- Log-phase *E. coli* (50 µL/6 mL culture medium)
- Log-phase *E. coli* (50 µL/6 mL culture medium) + 1 µg/mL IFN-γ

The supernatants were collected at 24 hours and measured.

Cell type	Stimulus	IL-12 (pg/mL)
THP-1	Neat	ND
THP-1	LPS	ND
THP-1	LPS + IFN-γ	41
THP-1	*E. coli*	ND
THP-1	*E. coli* + IFN-γ	170

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Explanation of symbols

Symbol	Description	Symbol	Description
REF	Catalogue Number	LOT	Batch code
RUO	Research Use Only	IVD	*In vitro* diagnostic medical device
	Use by		Temperature limitation
	Manufacturer	EC REP	European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
Protect from Light	Protect from light		Consult accompanying documents
i	Directs the user to consult instructions for use (IFU), accompanying the product.		

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Human IL-12 p70 Assay Summary

Add 50 µL Incubation Buffer or 50 µL Standard Diluent Buffer depending on the sample type

↓

Add 100 µL of standards, controls and samples

↓

Add 50 µL of Biotin Conjugate
Incubate for 3 hours at RT

↓ **aspirate and wash 4x**

Incubate 100 µL of Streptavidin-HRP Working Solution for 30 minutes at RT

↓ **aspirate and wash 4x**

Incubate 100 µL of Stabilized Chromogen for 30 minutes at RT in the dark

↓

Add 100 µL of Stop Solution and read at 450 nm

Total time: 4 hours