Human IL-12p70 ELISA Kit

Cat#: orb506700 (Product Manual)

1. Intended use

The Diaclone IL-12p70 ELISA kit is a solid phase sandwich ELISA for the in-vitro qualitative and quantitative determination of IL-12p70 in supernatants, buffered solutions or serum and plasma samples. This assay will recognise both natural and recombinant human IL-12p70.

This kit has been configured for research use only. Not suitable for use in therapeutic procedures.

2. Introduction

2.1. Summary

IL-12 is a potent regulator of cell mediated immune response produced by activated monocytes/macrophages cells, B lymphocytes and connective tissue type mast cells. The biologically active form of IL-12 is a 70 kDa heterodimeric glycoprotein consisting of disulfide-linked 35 kDa (p35) light chain and 40 kDa (p40) heavy chain subunits. The two subunits are genetically unrelated.

The p70 form is the only biologically active form of IL-12.

The p35 subunit has homology to IL-6, while p40 has homology with IL-23. IL-12 has been found to bind to IL-12R.IL-12R has been reported to be present on IL-2 activated CD4+, CD8+ and CD56+ cells.IL-12 exerts a variety of biological effects on human T and NK cells. IL-12 induces an IFN production and other cytokines from peripheral blood T and NK cells. Its role is directing development and proliferation of Th1 cells. Thus IL-12 is linked with autoimmunity, high level have also been reported for chronic inflammatory reactions, bacterial and viral infection.

2.2. Principle of the method

A capture Antibody highly specific for IL-12p70 has been coated to the wells of the microtitre strip plate provided during manufacture. Binding of IL-12p70 samples and known standards to the capture antibodies and subsequent binding of the biotinylated secondary antibody to the analyte is completed during the same incubation period. Any excess unbound analyte and secondary antibody is removed. The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing. A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of IL-12p70 present in the samples and standards. The absorbance of the colour complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a

standard curve. This standard curve can then be used to accurately determine the concentration of IL-12p70 in any sample tested.

3. Reagents provided and reconstitution

Reagents (Store@2-8^{O}C)	**Quantity 1x48 well kit** Cat no. 950.070.048	**Quantity 1x96 well kit** Cat no. 950.070.096	**Quantity 2x96 well kit** Cat no. 950.070.192	**Reconstitution**
96 well microtitre strip plate	1/2	1	2	Ready to use (Pre-coated)
Plastic plate covers	2	2	4	n/a
Standard: 200pg/ml	1	2	4	Reconstitute as directed on the vial (see reagent preparation, section 8)
Standard Diluent (Buffer)	1 (25ml)	1 (25ml)	1 (25ml)	10x Concentrate, dilute in distilled water (see reagent preparation, section 8)
Standard Diluent (Serum)	1 (7ml)	1 (7ml)	2 (7ml)	Ready to use
Control	1	2	4	Reconstitute as directed on the vial (see reagent preparation, section 8)
Biotinylated anti-IL-12p70	1 (0.4ml)	1 (0.4ml)	2 (0.4ml)	Dilute in biotinylated antibody diluent (see reagent preparation, section 8)
Biotinylated Antibody diluent	1 (7ml)	1 (7ml)	1 (13ml)	Ready to use
Streptavidin-HRP	1 (5µl)	2 (5µl)	4 (5µl)	Add 0.5ml of HRP diluent prior to use (see reagent preparation, section 8)
HRP Diluent	1 (23ml)	1 (23ml)	1 (23ml)	Ready to use
Wash Buffer	1 (10ml)	1 (10ml)	2 (10ml)	200x Concentrate dilute in distilled water (see reagent preparation, section 8)
TMB Substrate	1 (11ml)	1 (11ml)	1 (24ml)	Ready to use
H_2SO_4 stop reagent	1 (11ml)	1 (11ml)	2 (11ml)	Ready to use

4. Materials required but not provided

- Microtitre plate reader fitted with appropriate filters (450nm required with optional 620nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

5. Storage Instructions

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°C). Expiry of the kit and reagents is stated on box front labels. The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

6. Specimen collection, processing & storage

Cell culture supernatants, human serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.

Serum: Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. Following clotting, centrifuge at approximately 1000 x g for 10 min and remove serum.

Plasma: EDTA, citrate and heparin plasma can be assayed. Spin samples at 1000 x g for 30 min to remove particulates. Harvest plasma.

Storage: If not analyzed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

7. Safety & precautions for use

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g.CDC/NIH Health manual : "Biosafety in Microbiological and Biomedical Laboratories" 1984.

- Laboratory gloves should be worn at all times.
- Avoid any skin contact with H2SO4 and TMB. In case of contact, wash thoroughly with water.

- Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
- Do not pipette by mouth.

- When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottles labels.

- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use.

- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration.
- Cover or cap all reagents when not in use.
- Do not mix or interchange reagents between different lots.

- Do not use reagents beyond the expiration date of the kit.

- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of H_2SO_4 and substrate solution, avoid pipettes with metal parts.
- Use a clean plastic container to prepare the washing solution.
- Thoroughly mix the reagents and samples before use by agitation or swirling.

- All residual washing 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.

- The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB is toxic avoid direct contact with hands. Dispose off properly.

- If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbance's within 1 hour after completion of the assay.

- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- Follow incubation times described in the assay procedure.
- Dispense the TMB solution within 15 min of the washing of the microtitre plate.

8. Assay Preparation

Bring all reagents to room temperature before use

8.1. Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard, zero and control should be tested in duplicate. Remove sufficient Microwell Strips for testing from the aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8oC storage.

Example plate layout (example shown for a 6 point standard curve)

	Standards / Controls		Sample Wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	200	200										
B	100	100										
C	50	50										
D	25	25										
E	12.5	12.5										
F	6.25	6.25										
G	zero	zero										
H	Ctrl	Ctrl										

All remaining empty wells can be used to test samples in duplicate

8.2. Preparation of Wash Buffer

Dilute the (200x) wash buffer concentrate 200 fold with distilled water to give a 1x working solution. Pour entire contents (10 ml) of the Washing Buffer Concentrate into a clean 2,000 ml graduated cylinder. Bring final volume to 2,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle and store at 2˚-25˚C.

8.3. Preparation of Standard Diluent Buffer

Add the contents of the vial (10x concentrate) to 225 ml of distilled water before use.

This Solution can be stored at 2-8°C for up to 1 week.

8.4. Preparation of Standard

Depending on the type of samples you are assaying, the kit may include two standard diluents. Because biological fluids might contain proteases or cytokine-binding proteins that could modify the recognition of the cytokine you want to measure, you should reconstitute standard vials with the most appropriate Standard Diluent.

For serum and plasma samples: use Standard Diluent - Serum.

For cell culture supernatants: use Standard Diluent Buffer.

Standard vials must be reconstituted with the volume of standard diluent shown on the vial immediately prior to use. This reconstitution gives a stock solution of 200pg/ml of IL-12p70. Mix the reconstituted standard gently by repeated aspirations/ejections. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 200 to 6.25pg/ml. A fresh standard curve should be produced for each new assay.

- Immediately after reconstitution add 200µl of the reconstituted standard to well's A1 and A2, which provides the highest concentration standard at 200pg/ml.
- Add 100µl of appropriate standard diluent to the remaining standard wells B1 and B2 to F1 and F2.
- Transfer 100µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells.
- Continue this 1:1 dilution using 100µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 200pg/ml to 6.25pg/ml.
- Discard 100µl from the final wells of the standard curve (F1 and F2).

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred directly into the relevant wells.

8.5. Preparation of Controls

Freeze-dried control vials should also be reconstituted with the most appropriate Standard Diluent to your samples.

For serum and plasma samples: use Standard Diluent - Serum.

For cells culture supernatants: use Standard Diluent Buffer.

The supplied Controls must be reconstituted with the volume of Standard Diluent indicated on the vial. Reconstitution of the freeze-dried material with the indicated volume, will give a solution at the concentration stated on the vial. Do not store after use.

8.6. Preparation of Biotinylated anti-IL-12p70

It is recommended this reagent is prepared immediately before use. Dilute the biotinylated anti-IL-12p70 with the biotinylated antibody diluent in an appropriate clean glass vial using volumes appropriate to the number of required wells. Please see example volumes below:

Number of wells required	Biotinylated Antibody (µl)	Biotinylated Antibody Diluent (µl)
16	40	1060
24	60	1590
32	80	2120
48	120	3180
96	240	6360

8.7. Preparation of Streptavidin-HRP

It is recommended to centrifuge vial for a few seconds in a microcentrifuge to collect all the volume at the bottom.

Dilute the 5□l vial with 0.5ml of HRP diluent immediately before use. Do not keep this diluted vial for future experiments. Further dilute the HRP solution to volumes appropriate for the number of required wells in a clean glass vial. Please see example volumes below:

Number of wells required	Streptavidin-HRP (µl)	Streptavidin-HRP Diluent (ml)
16	30	2
24	45	3
32	60	4
48	75	5
96	150	10

9. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use.

Prepare all reagents as shown in section 8.

Note: final preparation of Biotinylated Secondary Antibody (section 8.6) and Streptavidin-HRP (section 8.7) should occur immediately before use.

Assay Step		Details
1.	Addition	**Prepare Standard curve** as shown in section 8.4 above
2.	Addition	Add 100µl of each**, Sample, Standard, Control and zero (appropriate standard diluent)** in duplicate to appropriate number of wells
3.	Addition	Add 50µl of diluted **biotinylated anti-IL-12p70** to all wells
4.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **3 hours**
5.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
6.	Addition	Add 100µl of **Streptavidin-HRP** solution into all wells
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **30 min**
8.	Wash	Repeat wash step 5.
9.	Addition	Add 100µl of ready-to-use **TMB Substrate Solution** into all wells
10.	Incubation	Incubate in the dark for **12-15 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.
11.	Addition	Add 100µl of **H_2SO_4:Stop Reagent** into all wells

Read the absorbance value of each well (immediately after step 11.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).

*Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range.

10. Data Analysis

Calculate the average absorbance values for each set of duplicate standards, controls and samples.

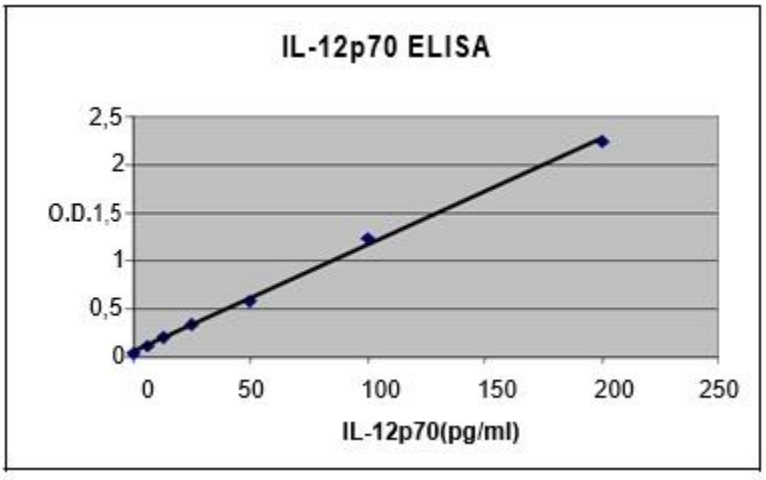
Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding IL-12p70 standard concentration on the horizontal axis.

The amount of IL-12p70 in each sample is determined by extrapolating OD values against IL-12p70 standard concentrations using the standard curve.

Example IL-12p70 Standard curve

Standard	IL-12p70 Conc	OD (450nm) mean	CV (%)
1	200	2.244	5.46
2	100	1.231	0.04
3	50	0.572	2.89
4	25	0.342	1.04
5	12.5	0.202	7.5
6	6.25	0.11	9.09
zero	0	0.028	2.37



Note: curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.

11. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample

buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.

Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore a fresh standard curve must be prepared and run for every assay.

12. Performance Characteristics

12.1. Sensitivity

The sensitivity, minimum detectable dose of IL-12p70 using this Diaclone IL-12p70 ELISA kit was found to be 2.2pg/ml. This was determined by adding 3 standard deviations to the mean OD obtained when the zero standard was assayed 40 times.

12.2. Specificity

The assay recognizes both natural and recombinant human IL-12p70. To define the specificity of this ELISA several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (IL-1□, IL-1□, IL-10, IL-12p40, IFN□, IL-4, IL-6, TNF□, IL-8 and IL-13).

12.3. Precision

Intra-assay

Reproducibility within the assay will be evaluated in three independent experiments. Each assay will be carried out with 6 replicates (3 duplicates) of 3 spiked serum or human pooled serum samples and 2 supernatants containing different concentrations of IL-12p70. The overall intra-assay coefficient of variation has been calculated to be 4.3%.

Session	Sample	Assay 1 IL-12p70 pg/ml	Assay 2 IL-12p70 pg/ml	Assay 3 IL-12p70 pg/ml	Mean IL-12p70 pg/ml	SD	CV %
1	1	303	310	281	298	15.1	5.08
	2	113	121	117	117	4.0	3.42
	3	72	69	98	69.7	2.1	2.99
	4	185	215	219	206.3	18.6	9.01
	5	108	113	114	111.7	3.2	2.88
2	1	283	278	272	277.7	5.5	1.98
	2	127	133	109	123	12.5	10.15
	3	70	64	66	66.7	3.1	4.58
	4	200	196	181	192.3	10.0	5.21
	5	109	111	110	110.0	1.0	0.91
3	1	279	273	283	278.3	5.0	1.81
	2	136	135	131	134	2.6	1.97
	3	64	67	66	65.7	1.5	2.33
	4	194	219	207	206.7	12.5	6.05
	5	107	104	96	102.3	5.7	5.56

Inter-assay

Assay to assay reproducibility within one laboratory will be evaluated in three independent experiments by two technicians. Each assay will be carry out with 6 replicates of 3 spiked serum human pooled serum samples and 2 supernatants containing different concentration of IL-12p70. The calculated overall coefficient of variation was 8.9%.

Technician	Session	Sample 1 IL-12p70 pg/ml	Sample 2 IL-12p70 pg/ml	Sample 3 IL-12p70 pg/ml	Sample 4 IL-12p70 pg/ml	Sample 5 IL-12p70 pg/ml
A	1	276	117	59	193	89
		268	105	57	191	88
		270	102	62	178	92
	2	283	100	49	197	87
		259	101	55	182	94
		245	110	63	166	81
	3	300	131	67	199	106
		286	137	70	219	109
		289	113	70	209	112
B	1	303	121	72	185	108
		310	117	69	215	113
		281	127	68	219	114
	2	283	133	70	200	109
		278	109	64	196	111
		272	127	66	181	110

	3	279 273 284	136 135 131	64 67 65	194 219 207	107 104 96
Mean IL-12p70 pg/ml		280	120	64	197	102
SD		15	14	6	15	11
CV %		5.5	11.3	9.4	7.8	10.4

12.4. Dilution Parallelism

Four human pooled serum samples with different levels of IL-12p70 were analysed at different serial two fold dilutions (1:2 To 1:8) with two replicates each. Recoveries ranged from 68 to 110% with an overall mean recovery of 95%.

12.5. Spike Recovery

The spike recovery was evaluated by spiking 3 concentrations of IL-12p70 in human serum in 2 separate experiments. Recoveries ranged from 86 to 94% with an overall mean recovery of 91%.

12.6. Stability

Storage Stability

Aliquots of spiked serum samples were stored at –20°C, 2-8°C, room temperature (RT) and at 37°C and the IL-12p70 level determined after 24h. We observed a significant loss of IL-12p70 immunoreactivity during storage at 2-8°C (19%), at RT (31%) and 37°C (34%)

Freeze-thaw Stability

Aliquots of spiked serum were stored frozen at –20°C and thawed up to 5 times and the IL-12p70 level was determined. There was a decrease in activity of IL-12p70 after 3 and 5 cycles of freezing and thawing.

12.7. Expected serum values

A panel of 24 human sera was tested for IL-12p70. All were below the detection level of 2.2pg/ml.

12.8. Standard Calibration

This immunoassay is calibrated against the International Reference Standard NIBSC 95/544. NIBSC 95/544 is quantitated in International Units, equivalence in pg/ml is indicated. 1pg NIBSC corresponding to 0.53pg Diaclone IL-12p70.

13. Assay Summary

Total procedure length: 3h45mn

Add 100 µl of sample and diluted standard/controls and 50µl Biotinylated anti-IL-12p70

↓

Incubate 3 hours at room temperature

↓

Wash three times

↓

Add 100µl of Streptavidin-HRP

↓

Incubate 30min at room temperature

↓

Wash three times

↓

Add 100µl of ready-to-use TMB Protect from light. Let the color develop for 12-15 mn. ↓

Add 100µl H_2SO_4

↓

Read Absorbance at 450 nm