



User's Manual

Mouse IL-12/IL-23 p40 ELISA

Sandwich Enzyme-Linked Immunosorbent Assay for the detection
of Mouse IL-12/IL-23 p40

REF

IB99557



96

Storage: 2-8°C

RUO

For research use only, not for use in diagnostic procedures.

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1. Introduction

Subunit beta of interleukin 12 (also known as natural killer cell stimulatory factor 2, or cytotoxic lymphocyte maturation factor 2, p40) (human gene name IL12B) is a subunit of human interleukin 12. This gene encodes a subunit of interleukin 12, a cytokine that acts on T and natural killer cells, and has a broad array of biological activities. Interleukin 12 is a disulfide-linked heterodimer composed of the 40 kD cytokine receptor like subunit encoded by this gene, and a 35 kD subunit encoded by IL12A. This cytokine is expressed by activated macrophages that serve as an essential inducer of Th1 cells development. This cytokine has been found to be important for sustaining a sufficient number of memory/effector Th1 cells to mediate long-term protection to an intracellular pathogen. Overexpression of this gene was observed in the central nervous system of patients with multiple sclerosis (MS), suggesting a role of this cytokine in the pathogenesis of the disease. The promoter gene polymorphism of this gene has been reported to be associated with the severity of atopic and non-atopic asthma in children. Editing causes a reduction in the extent of IL-12 signaling as the β subunit is involved in signal transduction. This leads to insufficient IFN- γ production, possibly due to an interruption of the binding of IL-12 to its receptor. Binding may be restricted due to a conformational change caused by the change of amino acid. The change in the signaling cascade and reduction of IFN- γ effects downregulation of IgE. Atrophy patients have enhanced IgE sensitivity to everyday environmental factors such as asthma. This is the only editing event linked to atrophy. Interleukin-12 p40 also serves as a subunit of Interleukin 23.

2. Principles of Method

The design of this assay is based on a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to Mouse IL-12/IL-23 p40. Samples are pipetted into these wells. Nonbound Mouse IL-12/IL-23 p40 and other components of the sample should be removed by washing, then monoclonal antibody specific to Mouse IL-12/IL-23 p40 added. In order to quantitatively determine the amount of Mouse IL-12/IL-23 p40 present in the sample, Avidin Horseradish Peroxidase (HRP) should be added to each microplate well. The final step, a TMB-substrate solution added to each well. Finally, a sulfuric acid solution is added and the resulting yellow colored product is measured at 450nm. Since the increases in absorbency is directly proportional to the amount of captured Mouse IL-12/IL-23 p40.

3. Intended Use

The IBL-AMERICA Mouse IL-12/IL-23 p40 ELISA kit is to be used for the determination of Mouse IL-12/IL-23 p40 in Mouse serum, Mouse plasma, cell lysate, culture supernatants and buffered solution. The assay will recognize native and recombinant Mouse IL-12/IL-23 p40.

This kit has been configured for research use only and is not to be used in diagnostic procedures.

4. Storage and Stability

All kit components of this kit are stable at 2 to 8°C. Any unused reconstituted standard should be discarded or frozen at -70°C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

5. Chemical Hazard

- Stop solution: This reagent is an irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothing. Wear suitable protective clothing, gloves and eye protection. In the event of contact with eyes or skin, wash immediately with plenty of water.
- Standard protein and 2nd Antibody containing Sodium Azide as a preservative.

6. Kit Contents

Contents	Number	Volume
96 Well Plate	1 (in aluminum foil bag with desiccant)	
Washing Buffer	2	(20X) 25 ml
Standard Protein	1 Glass vial (lyophilized)	
Standard/Sample Dilution Buffer	1	25 ml
Secondary Antibody	1 Glass vial (lyophilized)	
Avidin HRP(X100)	1	150 µl
Secondary antibody/ Avidin HRP Dilution Buffer	1	25 ml
Substrate (TMB)	1	15 ml
Stop Solution	1	15 ml
Protocol booklet	1	
Plate sealers	2	

- ① 96 Well Plate
: Mouse IL-12/IL-23 p40 microtiter plate, one plate of 96 wells (8 well strips x 12).
A plate using break-apart strips coated with a mouse monoclonal antibody specific to Mouse IL-12/IL-23 p40.
 - ② Standard Protein
: Recombinant Mouse IL-12/IL-23 p40.
 - ③ Secondary Antibody
: Biotinylated anti Mouse IL-12/IL-23 p40.
 - ④ Avidin HRP
: Avidin Horseradish Peroxidase (HRP, enzyme)
 - ⑤ Substrate (Stabilized chromogen)
: Tetramethylbenzidine (TMB) solution
 - ⑥ Stop Solution
: 1N solution of sulfuric acid (H₂SO₄).
 - ⑦ Plate sealer
: Adhesive sheet.
- Do not mix or interchange different reagents from various kit lots.

7. Materials Required But Not Provided

- ① Microtiter plate reader capable of measurement at or near 450nm.
- 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 Data analysis and graphing software
- ⑤ Vortex mixer
- ⑥ Polypropylene tubes for diluting and aliquoting standard
- ⑦ Absorbent paper towels
- ⑧ Calibrated beakers and graduated cylinders of various sizes

8. Reagent Preparation

1) Mouse IL-12/IL-23 p40 standard

1. Reconstitute the lyophilized Mouse IL-12/IL-23 p40 standard by adding 1 ml of *Standard/Sample Dilution Buffer* to make the 4 ng/ml standard stock solution. Allow solution to sit at RT for 5 minutes, then gently vortex to mix completely. Use within one hour of reconstituting

2. Prepare 1 ml of 1000 pg/ml top standard by adding 250 µl of the above stock solution in 750 µl of *Standard/Sample Dilution Buffer*. Perform 2-fold serial dilutions of the top standards to make the standard curve within the range of this assay (15.625 pg/ml ~ 1000 pg/ml) as below. *Standard/Sample Dilution Buffer* serves as the zero standard (0 pg/ml).

Standard	Add	Into
1000 pg/ml	250.0 µl of the std.(4 ng/ml)	750.0 µl of the <i>Standard/Sample Dilution Buffer</i>
500 pg/ml	500 µl of the std.(1000 pg/ml)	500.0 µl of the <i>Standard/Sample Dilution Buffer</i>
250 pg/ml	500 µl of the std.(500 pg/ml)	500.0 µl of the <i>Standard/Sample Dilution Buffer</i>
125 pg/ml	500 µl of the std.(250 pg/ml)	500.0 µl of the <i>Standard/Sample Dilution Buffer</i>
62.5 pg/ml	500 µl of the std.(125 pg/ml)	500.0 µl of the <i>Standard/Sample Dilution Buffer</i>
31.25 pg/ml	500 µl of the std.(62.5 pg/ml)	500.0 µl of the <i>Standard/Sample Dilution Buffer</i>
15.625 pg/ml	500µl of the std.(31.25 pg/ml)	500.0 µl of the <i>Standard/Sample Dilution Buffer</i>
0 pg/ml	1.0 ml of the <i>Standard/Sample Dilution Buffer</i>	

2) Secondary Antibody

100X secondary antibody solution can be made by adding 150 µl *Secondary antibody/Avidin HRP dilution buffer* in the vial.

1. Equilibrate to room temperature, mix gently.

2. Mix 20 μ l *Secondary Antibody concentrated solution* (100X) + 1.98 ml *Secondary antibody/Avidin HRP dilution buffer*. (Sufficient for two 8-well strip, prepare more if necessary)
Label as “Working Secondary antibody Solution”.
3. Return the unused *Secondary Antibody concentrated solution* to the refrigerator.

3) Avidin HRP(X100)

1. Equilibrate to room temperature, mix gently.
2. Mix 20 μ l *Avidin HRP concentrated solution* (100X) + 1.98 ml *Secondary antibody/Avidin HRP dilution buffer*. (Sufficient for two 8-well strip, prepare more if needed)
Label as “Working Avidin HRP Solution”.
3. Return the unused *Avidin HRP concentrated solution* to the refrigerator.

4) Washing buffer

1. Equilibrate to room temperature, mix to re-dissolve any precipitated salt.
2. Mix 0.5 volume *Wash buffer concentrate solution* (20X) + 9.5 volumes of deionized water. Label as “Working Washing Solution”.
3. Store both the concentrated and the Working Washing Solution in the refrigerator.

* Directions for washing

1. Fill the wells with 300 μ l of “Working Washing Buffer”.
Let soak for 1 to 3 minutes and then all residual wash-liquid must be drained from the wells by aspiration (taking care not to scratch the inside of the well) or decantation, followed by forceful tapping of the plate on absorbent paper. Never insert absorbent paper directly into the wells.
If using an automated washer, the operating instructions for washing equipment should be carefully followed.
2. Incomplete washing will adversely affects the assay and renders false results.
3. It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing to avoid strips coming free of the frame.

5) Sample preparation

Blood should be collected by venipuncture. For plasma samples, blood may be drawn into tubes containing sodium citrate or heparin, EDTA. The serum or plasma should be separated from the coagulated or packed cells by centrifugation. Samples may be shipped at room temperature and then stored refrigerated at 2-8°C if testing is to take place within one week after collection. If testing is to take place later than one week, samples should be stored at -20°C. Avoid repeated freeze/thawing.

9. Assay Procedure

- Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.
 - All standards, controls and samples should be run in duplicate for confirmation of reproducibility.
 - A standard curve must be run with each assay.
 - If particulate matter is present in the analyte, centrifuge or filter prior to analysis.
 - Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.
- 1) Determine the number of 16-well strips needed for assay. Insert these in the frame(s) for current use (Re-bag extra strips and frame. Refrigerate for further use).
 - 2) For the standard curve, add 100 µl of the standard to the appropriate microtiter wells. Add 100 µl of the *Standard/Sample/secondary antibody Dilution Buffer* to zero wells.
 - 3) Add 100 µl of samples or serum to each well.
 - 4) Cover the plate with the plate cover and incubate for 2 hours at 37°C.
 - 5) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See “Directions for washing”).
 - 6) Pipette 100 µl of “Working Secondary Antibody Solution” into each well.
 - 7) Cover the plate with the plate cover and incubate for 1 hour at 37°C.
 - 8) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See “Directions for washing”).
 - 9) Add 100 µl “Working Avidin HRP Solution” to each well.
 - 10) Cover the plate with the plate cover and incubate for 30 minutes at 37°C.
 - 11) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See “Directions for washing”).
 - 12) Add 100 µl of *Substrate* to each well. The liquid in the wells should begin to turn blue.
 - 13) Incubate the plate at room temperature.
 - Do not cover the plate with aluminum foil, or color may develop. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. O.D. values should be monitored and the substrate reaction stopped before O.D. of the positive wells exceeds the limits of the instrument. O.D. values at 450nm can only be read after the Stop Solution has been added to each well.
 - Because the *Substrate* is light sensitive, avoid the remained *Substrate* solution prolonged exposure to light.

- Typically, reaction is stopped 5~10 minutes after treatment of Substrate, but this time can be adjusted as the user desires.
- 14) Add 100 µl of *Stop Solution* to each well. The solution in the wells should change from blue to yellow.
 - 15) Read the absorbance of each well at 450nm. Read the plate within 20 minutes of adding the *Stop Solution*.
 - 16) Plot on graph paper the absorbance of the standard against the standard concentration (Optimally, the background absorbance can be subtracted from all data points, including standards, unknowns and controls, prior to plotting.). Draw a smooth curve through these points to construct the standard curve.
 - 17) Read the Mouse IL-12/IL-23 p40 concentrations for the unknown samples and controls from the standard curve plotted in step 18. Multiply value(s) obtained for the unknown sample by the dilution factor (Samples producing signals greater than that of the highest standard should be further diluted in the *Standard/Sample Dilution Buffer*).

10. Characteristics

1) Typical result

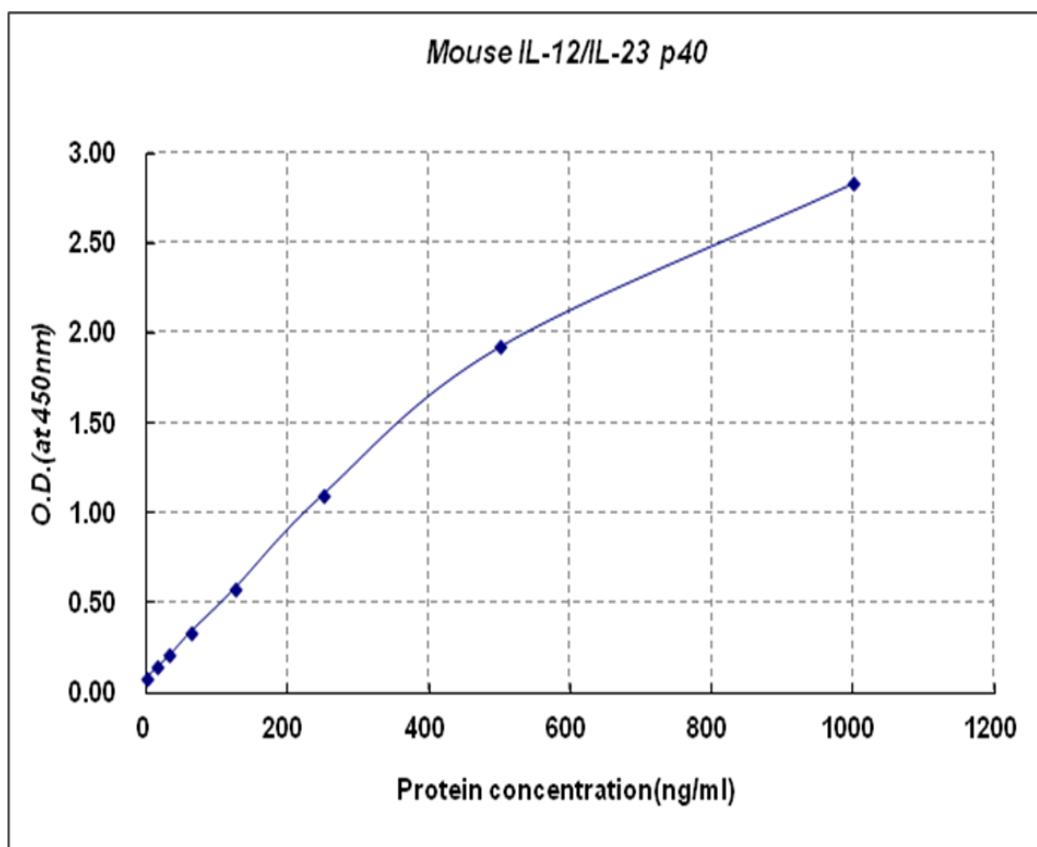
The standard curve below is for illustration only and **should not be used** to calculate results in your assay.

A standard curve must be run with each assay.

Standard Mouse IL-12/IL-23 p40 (pg/ml)	Optical Density (at 450nm)
0	0.083
15.615	0.145
32.15	0.205
62.5	0.337
125	0.580
250	1.101
500	1.921
1000	2.829

< Limitations >

- Do not extrapolate the standard curve beyond the 1000 pg/ml standard point.
- Other buffers and matrices have not been investigated.
- The rate of degradation of native Mouse IL-12/IL-23 p40 in various matrices has not been investigated.



(TMB reaction incubate at room temperature for 5 min)

2) Sensitivity

The minimal detectable dose of Mouse IL-12/IL-23 p40 was calculated to be 2.898 pg/ml, by subtracting three standard deviations from the mean of 12 zero standard replicates (ELISA buffer, S0) and intersecting this value with the standard curve obtained in the same calculation.

N	1	2	3	4	5	6	7	8	9	10	11	12
ZERO	0.122	0.125	0.120	0.124	0.136	0.122	0.112	0.133	0.118	0.135	0.117	0.134

Average	SD	LLD	LLD mean(pg/ml)
0.125	0.008	0.149	2.898

3) Specificity

The following substances were tested and found to have no cross-reactivity: Human IL-12/IL-23 p40, Rat IL-12/IL-23 p40.

4) Precision

① Within-Run (Intra-Assay)

(n=12)

Mean (pg/ml)	SD (pg/ml)	CV (%)
123.417	5.181	4.198
249.131	11.734	4.710
502.273	36.307	7.228
1000.63	67.881	6.784

② Between-Run (Inter-Assay)

(n=12)

Mean (pg/ml)	SD (pg/ml)	CV (%)
129.507	4.742	3.662
241.998	17.694	7.312
507.099	50.258	9.853
1001.06	92.932	9.283

5) Recovery

Recovery on addition is 87.316~96.764% (mean 92.509%)

Diluted serum(450nm)	Diluted Serum(450nm)+ 31.25 analyte (pg/ml) (450nm)	Diluted Serum + 31.25 added analyte (pg/ml) (450nm)	Recovery (%)
60.479	92.168	83.351	90.434
117.073	148.762	129.893	87.316
235.005	266.694	254.751	95.522
491.469	523.158	506.228	96.764

11. Troubleshooting

Problem	Possible Cause	Solution
High signal and background in all wells	• Insufficient washing	• Increase number of washes • Increase time of soaking between in wash
	• Too much Avidin-HRP	• Check dilution, titration
	• Incubation time too long	• Reduce incubation time
	• Development time too long	• Decrease the incubation time before the stop solution is added
No signal	• Reagent added in incorrect order, or incorrectly prepared	• Review protocol
	• Standard has gone bad (If there is a signal in the sample wells)	• Check the condition of stored standard
	• Assay was conducted from an incorrect starting point	• Reagents allows to come to 20~30°C before performing assay
Too much signal – whole plate turned uniformly blue	• Insufficient washing –unbound AV-HRP remaining	• Increase number of washes carefully
	• Too much Avidin -HRP	• Check dilution
	• Plate sealer or reservoir reused, resulting in presence of residual Avidin -HRP	• Use fresh plate sealer and reagent reservoir for each step
Standard curve achieved but poor discrimination between point	• Plate not developed long enough	• Increase substrate solution incubation time
	• Improper calculation of standard curve dilution	• Check dilution, make new standard curve
No signal when a signal is expected, but standard curve looks fine	• Sample matrix is masking detection	• More diluted sample recommended
Samples are reading too high, but standard curve is fine	• Samples contain protein levels above assay range	• Dilute samples and run again
Edge effect	• Uneven temperature around work surface	• Avoid incubating plate in areas where environmental conditions vary • Use plate sealer

12. Reference

- 1) "Entrez Gene: *IL12B* interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)".
- 2) Kondo N, Matsui E, Kaneko H, et al. (March 2004). "RNA editing of interleukin-12 receptor beta2, 2451 C-to-U (Ala 604 Val) conversion, associated with atopy". *Clin. Exp. Allergy* 34 (3): 363–8.
- 3) Oppmann, B; Lesley R, Blom B, Timans J C, Xu Y, Hunte B, Vega F, Yu N, Wang J, Singh K, Zonin F, Vaisberg E, Churakova T, Liu M, Gorman D, Wagner J, Zurawski S, Liu Y, Abrams J S, Moore K W, Rennick D, de Waal-Malefyt R, Hannum C, Bazan J F, Kastelein R A (November 2000). "Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12". *Immunity (UNITED STATES)* 13 (5): 715–25.

◆ Ordering Information

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