Human IL-12p40 ELISA Kit

Instructions for use

Catalogue numbers:

1x48 tests: EA102160
1x96 tests: EA102161
2x96 tests: EA102162

For research use only

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Human IL-12p40 ELISA KIT

1. Intended use

The OriGene IL-12p40 ELISA kit is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of IL-12p40 in supernatants, buffered solutions or serum and plasma samples. This assay will recognise both natural and recombinant human IL-12p40 monomer but also when in heterodimer with the p35 protein (IL-12p70) or with the p19 protein (IL-23).

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. The p40 subunit has been found to be expressed in a higher excess over p70.

IL-12 has been found to bind to IL-12R. The p40 subunit can also form a homodimer which has been shown to bind IL-12R and thus acts as an IL-12 antagonist 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 IFNγ 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-12p40 has been coated to the wells of the microtitre strip plate provided during manufacture. Binding of IL-12p40 samples and known standards to the capture antibodies and subsequent binding of the biotinylated anti-IL-12p40 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-12p40 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-12p40 in any sample tested.

3. Reagents provided and reconstitution

Reagents (Store@2-8°C)	**Quantity 1x48 well kit** Cat no. EA102160	**Quantity 1x96 well kit** Cat no. EA102161	**Quantity 2x96 well kit** Cat no. EA102162	**Reconstitution**
96 well microtitrer strip plate	1/2	1	2	Ready to use (Pre-coated)
Plastic plate covers	2	2	4	n/a
Standard: 2000 pg/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-12p40	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 H_2SO_4 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 pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8^{o}C 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	2000	2000										
B	1000	1000										
C	500	500										
D	250	250										
E	125	125										
F	62.5	62.5										
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 Wash 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 225ml 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 2000 pg/ml of IL-12p40. Mix the reconstituted standard gently by aspirations/ejections. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 2000 to 62.5 pg/ml. A fresh standard curve should be produced for each new assay.

- Immediately after reconstitution add 200µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 2000 pg/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 2000 pg/ml to 62.5 pg/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-12p40

It is recommended this reagent is prepared immediately before use. Dilute the biotinylated anti-IL-12p40 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 5µl 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 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-12p40** to all wells
4.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour**
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 wavelength (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

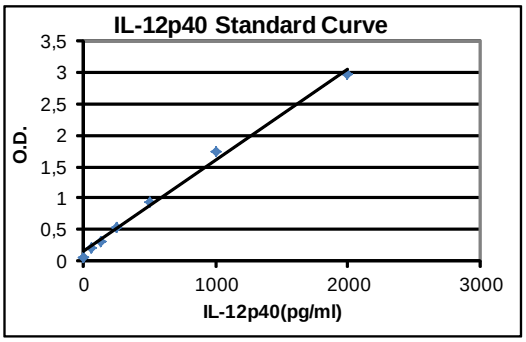
Calculate the average absorbance values for each set of duplicate standards, controls and samples. Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding IL-12p40 standard concentration on the horizontal axis.

The amount of IL-12p40 in each sample is determined by extrapolating OD values against IL-12p40 standard concentrations using the standard curve.

Example IL-12p40 Standard curve

Standard	IL-12p40 Conc pg/ml	OD (450nm) mean	CV (%)
1	2000	2.968	1.64
2	1000	1.737	7.16
3	500	0.942	6.15
4	250	0.536	1.65
5	125	0.317	5.95
6	62.5	0.196	12.28
zero	0	0.063	38.81



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-12p40, using this OriGeneIL-12p40 ELISA kit, was found to be **20 pg/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-12p40. 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-1α, IL-1β, IL-10, IL-2, IFNγ, IL-4, IL-6, TNFα, IL-8 and IL-13).
The assay will also recognize the p40 when in heterodimer with the p35 protein (IL-12p70) or with the p19 protein (IL-23).

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-12p40. **The overall intra-assay coefficient of variation has been calculated to be 3.7%.**

Session	Sample	Assay 1 IL-12p40 pg/ml	Assay 2 IL-12p40 pg/ml	Assay 3 IL-12p40 pg/ml	Mean IL-12p40 pg/ml	SD	CV %
1	1	1932	2048	2051	2010	68	3.38
	2	1021	1009	960	997	32	3.24
	3	479	470	473	474	5	0.97
	4	1307	1377	1258	1314	60	4.55
	5	676	664	723	688	31	4.53
2	1	2331	2321	2383	2345	33	1.42
	2	1185	1308	1270	1254	63	5.02
	3	524	506	503	511	11	2.22
	4	1509	1623	1605	1579	61	3.88
	5	843	793	892	843	50	5.87
3	1	2295	2138	2185	2206	81	3.65
	2	1143	1181	1141	1155	23	1.95
	3	541	500	483	508	30	5.87
	4	1499	1477	1394	1457	55	3.8
	5	876	782	817	825	48	5.76

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 or human pooled serum samples and 2 supernatants containing different concentration of IL-12p40. **The calculated overall coefficient of variation was 9.4%.**

Technician	Session	Sample 1 IL-12p40 pg/ml	Sample 2 IL-12p40 pg/ml	Sample 3 IL-12p40 pg/ml	Sample 4 IL-12p40 pg/ml	Sample 5 IL-12p40 pg/ml
A	1	2426	1213	487	1493	943
		2348	1286	505	1555	945
		2355	1250	542	1351	864
	2	2493	1333	397	1638	1029
		2389	1230	398	1726	931
		2540	1300	506	1579	786
	3	2222	1223	358	1469	910
		2219	1280	477	1417	714
		2302	1243	469	1514	687
B	1	1932	1021	479	1307	676
		2048	1009	470	1377	664
		2051	960	473	1258	723
	2	2331	1185	542	1509	843
		2321	11307	506	1623	793
		2383	1270	503	1605	892
	3	2295	1143	541	1499	876
		2138	1181	500	1477	782
		2185	1141	483	1394	817
Mean IL-12p40 pg/ml		2277	1199	479	1488	826
SD		160	108	49	123	106
CV %		7.0	9.0	10.3	8.3	12.8

12.4. Dilution Parallelism

Four human pooled serum samples with different levels of IL-12p40 were analysed at different serial two fold dilutions (1:2 To 1:8) with two replicates each. Recoveries ranged from 86 to 118% with an overall **mean recovery of 100%.**

12.5. Spike Recovery

The spike recovery was evaluated by spiking 3 concentrations of IL-12p40 in human serum in 2 separate experiments. Recoveries ranged from 99 to 110% with an overall **mean recovery of 103%.**

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-12p40 level determined after 24h. We observed no significant loss of IL-12p40 immunoreactivity during storage.

Freeze-thaw Stability

Aliquots of spiked serum were stored frozen at –20°C and thawed up to 5 times and the IL-12p40 level was determined. There was no decrease in activity of IL-12p40 after cycles of freezing and thawing.

12.7. Expected serum values

A panel of 24 human sera was tested for IL-12p40. 3 are below the detection level, 21 are ranged from 65 to 483 pg/ml with a mean at 187 pg/ml and a SD at 126 pg/ml.

13. OriGeneHuman IL-12p40 ELISA references

Beyth S. et al., Blood, 2005; 105(5): 2214 - 2219
Braitch, M. et al., Arch Neurol., 2008; 65(5): 633-635
Rodriguez-Zapata, M. et al., Infect. Immun., 2010; IAI.01385-09
Tenca C. et al., J. Immunol., 2005; 174(11): 6757 - 6763

14. Assay Summary

Total procedure length: 1h45mn

Add 100 µl of sample and diluted standard/controls
and 50 µl Biotinylated anti-IL-12p40

↓

Incubate 1 hour at room temperature

↓

Wash three times

↓

Add 100µl of Streptavidin-HRP

↓

Incubate 30min at room temperature

↓

Wash three times

↓

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

↓

Add 100 µl of H_2SO_4 Stop reagent

↓

Read Absorbance at 450 nm

TECHNICAL CONSULTATION

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Not for use in diagnostic procedures

15. International Summaries

15.1. French

PREPARATION DES REACTIFS : RESUME

1.Tampon de Lavage *(Wash buffer)*

Ajouter 10 ml de **Tampon de Lavage concentré** 200 fois (*Wash Buffer Concentrate 200X*) à 1990 ml d'eau distillée

2. Tampon de Dilution du Standard *(Standard Diluent Buffer)*

Ajouter 25 ml de **Tampon de Dilution du Standard concentré** 10 fois (*Standard Diluent Buffer Concentrate 10X)* à 225 ml d'eau distillée

3. Standard IL-12p40 *(IL-12p40 Standard)*

Reconstituer le **Standard IL-12p40** en ajoutant le volume indiqué sur le flacon de **Tampon approprié** (Tampon de Dilution du Standard ou Tampon de Dilution du Standard : Sérum)

4. Contrôles *(IL-12p40 Control)*

Reconstituer les **Contrôles** (*Control*) en ajoutant le volume indiqué sur le flacon avec le **Tampon approprié** (Tampon de Dilution du Standard ou Tampon de Dilution du Standard : Sérum)

5. Anti-IL-12p40 Biotinylé *(Biotinylated anti-IL-12p40)*

Nombre de barrettes	**Anti-IL-12p40 Biotinylé** Concentré (µl)	**Diluent de l'Anticorps Biotinylé** (µl)
2	40	1060
3	60	1590
4	80	2120
6	120	3180
12	240	6360

6. Streptavidine-HRP *(Streptavidin-HRP)*

Nombre de barrettes	**Streptavidine-HRP pré-diluée** (µl)	**Diluent HRP** (ml) (*HRP-Diluent*)
2	30	2
3	45	3
4	60	4
6	75	5
12	150	10

RESUME DU PROTOCOLE OPERATOIRE: durée totale : 1h45mn

1. Ajouter 100 µl de Tampon de Dilution du Standard *(Standard Diluent Buffer)* approprié, en duplicat, dans les puits Standards (B1 à F2).
2. Ajouter à la pipette 200 µl de Standard IL-12p40 *(IL-12p40 Standard)* reconstitué dans les puits A1 et A2 puis réaliser des dilutions du Standard allant de 2000 à 62.5 pg/ml en transférant 100 µl d'un puit à l'autre. Jeter les 100 µl des derniers puits (F1 et F2).
3. Ajouter 100 µl de Tampon de Dilution du Standard approprié *(Standard Diluent Buffer ou Standard Diluent : Serum)* en duplicat dans les puits "blancs".
4. Ajouter 100 µl d'échantillon *(Sample)*, en duplicate, dans les puits désignés et 100 µl de contrôle *(IL-12p40 Control)*, en duplicat dans les puits contrôles.
5. Préparer l'anticorps anti IL-12p40 Biotinylé *(Biotinylated anti IL-12p40)*.
6. Ajouter 50 µl d'anticorps anti IL-12p40 Biotinylé dilué (diluted biotinylated anti IL-12p40□) dans tous les puits.
7. Couvrir les barrettes de puits et incuber pendant 1 heure à température ambiante (18-25°C).
8. Vider et laver les puits 3 fois avec le Tampon de Lavage *(Wash Buffer)*.
9. Préparer la Streptavidine-HRP.
10. Ajouter 100 µl de Streptavidine-HRP diluée (diluted Streptavidin-HRP) dans tous les puits.
11. Couvrir les puits et incuber pendant 30 minutes à température ambiante (18°-25°C).
12. Vider et laver les puits 3 fois avec le Tampon de Lavage *(Wash Buffer).*
13. Ajouter 100 µl de solution de TMB (*TMB solution*) prête à l'emploi dans tous les puits y compris les "blancs".
14. Incuber pendant environ 12-15 minutes à température ambiante (18°-25°C) à l'obscurité.
15. Ajouter 100 µl d'H_2SO_4: Solution Stop *(H_2SO_4 : Stop Reagent)* dans tous les puits y compris les "blancs".
16. Mesurer l'absorbance à la longueur d'onde 450 nm et optionnellement à 620 nm (entre 610 et 650 nm) comme longueur d'onde de référence.

Remarque: Une valeur de Densité Optique supérieure à la gamme de la courbe Standard peuvent engendrer des résultats de taux d'IL-12p40 incorrects dans les échantillons testés. C'est pourquoi, il est recommandé de diluer de tels échantillons avec le Tampon de Dilution du Standard *(Standard Diluent Buffer)* **afin de quantifier précisément le véritable taux d'IL-12p40.**

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15.2. Spanish

PREPARACIÓN DE LOS PRODUCTOS

1. Tampón de Lavado *(Wash Buffer)*	Añadir 10 ml de **Tampón de Lavado Concentrado** 200 X (*Wash Buffer Concentrate 200X*) a 1990 ml de agua destilada.
2 Tampón diluyente del estándar *(Standard Diluent Buffer)*	Añadir 25 ml de **Tampón Diluyente del Estándar Concentrado** 10 X (*Standard Diluent Buffer Concentrate 10X)* a 225 ml de agua destilada.
3. Estándar IL-12p40 *(IL-12p40 Standard)*	Reconstituir el **Estándar IL-12p40,** como indica la etiqueta del vial, añadiendo el Diluyente del Estándar apropiado *(Standard Diluent Buffer / Standard diluent Buffer: Serum)*
4. Controles *(Control IL-12p40)*	Reconstituir los **controles,** como indica la etiqueta del vial, añadiendo el Diluyente del Estándar apropiado *(Standard Diluent Buffer / Standard diluent Buffer: Serum)*

5.Anti-IL-12p40 biotinilado *(Biotinylated anti-IL-12p40)*

Número de tiras	**Anticuerpo biotinilado** concentrado (µl)	**Diluyente del anticuerpo biotinilado** (µl)
2	40	1,060
3	60	1,590
4	80	2,120
6	120	3,180
12	240	6,360

6. Estreptavidina-HRP *(Streptavidin-HRP)*

Número de tiras	**Estreptavidina-HRP** prediluida (µl)	**Diluyente de HRP** (ml)
2	30	2
3	45	3
4	60	4
6	75	5
12	150	10

RESUMEN DEL PROTOCOLO. El procedimiento total tiene una duración de 1h45min.

1. Añadir 100 µl del Tampón Diluyente del Estándar apropiado (Standard diluent buffer), por duplicado, a los pocillos designados para el estándar (B1 to F2).
2. Pipetear 200 µl del Estándar IL-12p40 (IL-12p40 Standard) reconstituido en los pocillos A1 y A2 y hacer diluciones seriadas del estándar con el rango de concentraciones de 2000 al 62.5 pg/ml, transfiriendo 100 µl de un pocillo al siguiente. Descartar 100 µl de los últimos pocillos.
3. Añadir 100 µl del Tampón Diluyente del Estándar apropiado (Standard diluent buffer), por duplicado, a los pocillos que van a ser el "blanco".
4. Añadir 100 µl de las muestras (Sample), por duplicado, a los pocillos designados para ello, y 100 µl del Control reconstituido (IL-12p40 Control), por duplicado, a los pocillos designados como "control".
5. Preparar el anticuerpo Anti-IL-12p40 Biotinilado (Biotinlyated anti-IL-12p40).
6. Añadir 50 µl del anti-IL-12p40 Biotinilado y diluido, a todos los pocillos.
7. Cubrir la placa e incubar durante 1 horas a temperatura ambiente (18-25°C).
8. Vaciar y lavar la placa 3 veces con Tampón de Lavado (Wash Buffer).
9. Preparar la Estreptavidina-HRP (Streptavidin-HRP).
10. Añadir 100 µl de Estreptavidina-HRP diluida a todos los pocillos.
11. Cubrir la placa e incubar 30 minutos a temperatura ambiente (18° to 25°C).
12. Vaciar y lavar la placa 3 veces con Tampón de Lavado.
13. Añadir 100 µl de solución TMB preparado para utilizar (TMB Solution), a todos los pocillos, incluidos los pocillos con "blancos".
14. Incubar la placa durante 12-15 minutos a temperatura ambiente (18° to 25°C) y en oscuridad.
15. Añadir 100 µl de H_2SO_4: Solución de Parada (H_2SO_4: Stop Reagent) a todos los pocillos, incluidos los pocillos con los "blancos".
16. Medir la intensidad de color a 450 nm y a 620 nm como longitud de onda de referencia (de 610 nm a 650 nm sería aceptable).
17.

Nota: El cálculo de concentraciones de muestras con densidad óptica que supere el rango de la curva estándar, resultaría incorrecto, dando niveles de IL-12p40 más bajos de lo real. Estas muestras, requerirían ser diluidas con el Tampón de Dilución de Estándar adecuado, para poder precisar la cantidad real de IL-12p40.