Human IP-10 ELISA Kit

Instructions for use

Catalogue numbers: 1x48 tests:EA101265
1x96 tests:EA101266
2x96 tests:EA101267

For research use only

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Human IP-10 ELISA KIT

1. Intended use

The OriGene IP-10 ELISA kit is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of IP-10 (Interferon-gamma inducibleProtein 10kDa) also known as CXCL10 in supernatants, buffered solutions or serum and plasma samples. This assay will recognise both natural and recombinant human IP-10.

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

2. Introduction

2.1. Summary

IP-10 (Interferon-gamma inducible Protein 10kDa) also known as CXCL10, is secreted by several cell types in response to IFNγ and LPS. These cell types include monocytes, endothelial cells and fibroblasts.(1).The gene for IP-10 is located on chromosome 4 in a cluster among several other cytokines and encodes a 98 amino acid precursor protein.(1).
IP-10 has been attributed to several roles, such as chemoattraction for monocytes and T cells (but not for neutrophils), inhibition of bone marrow colony formation and angiogenesis, promotion of T cells adhesion molecule expression (2) (3).
IP-10 shares a common receptor, CXCR3, with the chemokine MIG, but has been shown to play a distinct role in host defense in infections.(4)
IP-10 expression has been associated with HIV infection (5), is involved in inflammatory skin disease (6) and other allergic diseases; it appears in inflammation of the nervous system and in Alzheimer's disease (astrocytes expressing IP-10 are commonly associated with senile plaques) (7).

2.2. Principle of the method

A capture Antibody highly specific for IP-10 has been coated to the wells of the microtitre strip plate provided during manufacture. Binding of IP-10 in samples and known standards to the capture antibodies is completed and then any excess unbound analyte is removed. During the next incubation period the binding of the biotinylated anti-IP-10secondary antibody to the analyte occurs. Any excess unbound secondary antibody is then 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 IP-10 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 IP-10 in any sample tested.

3. Reagents provided and reconstitution

Reagents (Store@2-8^{o}C)	**Quantity 1x48 well kit** Cat no.EA101265	**Quantity 1x96 well kit** Cat no.EA101266	**Quantity 2x96 well kit** Cat no.EA101267	**Reconstitution**
96 well microtiter strip plate	1/2	1	2	Ready to use (Pre-coated)
Plastic plate covers	2	2	4	n/a
Standard:200 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)
Biotinylated anti-IP10	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 and1,000µl 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 2and 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, incase 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 theassay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting andseparation.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 x g for10 min.
Serum: Use pyrogen/endotoxin freecollecting 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 removeparticulates. Harvest plasma.
Storage: If not analyzed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated freeze-thawcycles 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
- Allreagents 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 fromdeterioration
- 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 bytapping 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 withmetal 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 beencontaminated 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 incubationtimes 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 appropriatenumber of wells needed for running zeros and standards. Each sample, standard, zero and controlshould be tested**in duplicate**. Remove sufficient Microwell Strips for testing from the pouch immediately prior to use. Return any wells not required for thisassay 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		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												

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 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

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 IP-10.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 wells A1 and A2, which provides the highest concentration standard at 200pg/ml
- Add 100µl of 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 immediatelytransferred directly into the relevant wells.

8.5. Preparation of Samples

It is recommended to dilute human serum and plasmas 1:2 before assayingin Standard Diluent

8.6. Preparation of Biotinylated anti-IP-10

It is recommended this reagent is prepared immediately before use. Dilute the biotinylated anti-IP10 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
2.	Addition	Add 100µl of each**, diluted sample and zero (standard diluent)** in duplicate to appropriate number of wells
3.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **2 hour(s)**
4.	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
5.	Addition	Add 50µl of diluted **biotinylated anti-IP-10** to all wells
6.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour(s)**
7.	Wash	Repeat wash step 4.
8.	Addition	Add 100µl of **Streptavidin-HRP** solution into all wells
9.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **30 min**
10.	Wash	Repeat wash step 4.
11.	Addition	Add 100µl of ready-to-use **TMB Substrate Solution** into all wells
12.	Incubation	Incubate in the dark for **10-20 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.
13.	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

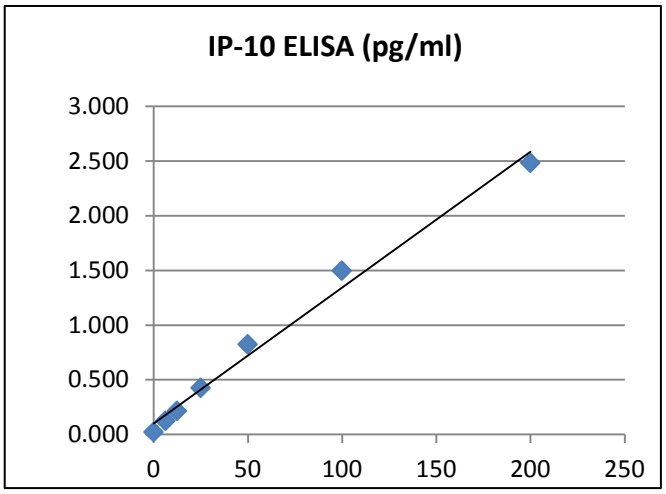
Calculate the average absorbance values for each set of duplicate standards, controls and samples**.**Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding IP-10 standard concentration on the horizontal axis.

The amount of IP-10 in each sample is determined by extrapolating OD values against IP-10standard concentrations using the standard curve**.**

Example IP-10 Standard curve

Standard	IP-10 Conc pg/ml	OD (450nm) Mean	CV (%)
1	200	2.484	2.5
2	100	1.498	0.5
3	50	0.825	1.2
4	25	0.426	3.2
5	125.5	0.217	1.3
6	6.25	0.126	0.6
Zero	0	0.022	-



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.

For serum and plasma samples which have been diluted according to the protocol (1:2), the calculated concentration should be multiplied by the dilution factor (x2).

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**afresh standard curve must be prepared and run for every assay.**

12. Performance Characteristics

12.1. Sensitivity

The sensitivity, minimum detectable dose of IP-10 using this OriGeneIP-10ELISA kit was found to be **5.7pg/ml**. This was determined by adding 3 standard deviations to the mean OD obtained when the zero standard was assayed 32 times.

12.2. Precision

	Intra Assay					Inter assay			
Sample	**n**	**Mean (pg/mL)**	**SD**	**CV%**	**Sample**	**n**	**Mean (pg/mL)**	**SD**	**CV%**
A	6	60.1	0.9	1.6	**A**	18	56.9	2.7	4.7
B	6	32.4	0.5	1.5	**B**	18	29.9	2.0	6.5
C	6	19.6	0.3	1.3	**C**	18	17.7	1.5	8.5

12.3. Recovery

The spike recovery was evaluated by spiking different concentrations of recombinant IP-10 in human serum. Recoveries ranged from 118.7% to 122.4%

12.4. Sample Stability

Aliquots of spiked serum samples were stored at -20°C and thawed up to five times : there was no significant loss of IP-10 activity. Aliquots of spiked serum samples were stored at -20°C, +4°C, Room temperature and 37°C and the IP-10 level was determined after 24h. There was no significant loss of IP-10 during storage under above conditions.

12.5. Expected values

21 sera from apparently healthy donors were evaluated for the presence of IP-10 in this assay. The detected human IP-10 levels ranged between 61 and 177pg/ml with a mean level of 106.5pg/ml and a standard deviation of ±28.6pg/ml. The normal levels measured may vary with sample collective used.

13. Bibliography

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14. References

Than, T. P. et al., Invest. Ophthalmol. Vis. Sci.,2011;52(6):3807
Correlations between Tear IP-10 and other Biomarkers in Normal and Dry Eye Patients

15. Assay Summary

Total procedure length : 3h45mn

Add sample and diluted standard

↓

Incubate 2 hours at room temperature

↓

Wash three times

↓

Add 50µl of biotinylated detection antibody

↓

Incubate 1 hour 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 10-20 mn.

↓

Add 100µl H_2SO_4

↓

Read Absorbance at 450 nm

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