Murine IP-10 ELISA Kit

Instructions for use

Catalogue numbers: 1x96 tests: 660.120.096

For research use only

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Murine IP-10 ELISA KIT

1. Intended use

The Murine IP-10 ELISA kit is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of murine IP-10 in supernatants, buffered solutions or serum and plasma samples. This assay will recognise both natural and recombinant murine IP-10.

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

2. Introduction

2.1. Summary

IP-10 is a member of the CXC subfamily of chemokines expressed by monocytes. It plays a pivotal role in immune system development and deployment. The Interferon-gamma inducible protein -10kD (IP-10) displays pleiotropic functions including stimulation of monocytes, natural killer and T-cell migration, regulation of T-cell and bone marrow progenitor maturation, modulation of adhesion molecule expression as well as inhibition of angiogenesis.

Through this inhibitory effect on neovascularization IP-10 exhibits anti-tumoral effects. It shares a common receptor, CXCR3, with the chemokine MIG, but has been shown to play a distinct role in host defense in infections. IP-10 is further involved in allergic contact dermatitis reactions and other allergic diseases. It appears during immune-mediated processes, in inflammation of the nervous system and in Alzheimer's disease.

Its involvement in the regulation of fibroproliferation following inflammatory lung injury has been described.

2.2. Principle of the method

A capture Antibody highly specific for mIP-10 has been coated to the wells of the microtiter strip plate. Binding of mIP-10 samples and known standards to the capture antibodies and subsequent binding of the biotin-conjugated anti-mIP-10 antibody to the analyte is completed during the same incubation period. Any excess unbound analyte and biotin-conjugated 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 stop reagent turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of mIP-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 mIP-10 in any sample tested.

3. Reagents provided and reconstitution

Reagents (Store@2-8°C)	**Quantity 1x96 wells kit** Cat no. 660.120.096	**Reconstitution**
96 well microtiter strip plate	1	Ready to use (Pre-coated)
Plastic plate covers	2	n/a
mIP-10 Standard: 1000 pg/ml	2	Reconstitute as directed on the vial (see Assay preparation, section 8)
Sample Diluent	1 (12ml)	Ready to use
Biotin conjugate anti-mIP-10	1 (70µl)	Dilute in Assay Buffer 1X (see Assay preparation, section 8)
Streptavidin-HRP	1 (150µl)	Dilute in Assay Buffer 1X (see Assay preparation, section 8)
Assay Buffer concentrate	1 (5ml)	20x Concentrate dilute in distilled water (see Assay preparation, section 8)
Wash Buffer concentrate	1 (50ml)	20x Concentrate dilute in distilled water (see Assay preparation, section 8)
Substrate Solution	1 (15ml)	Ready to use
Stop Solution	1 (15ml)	Ready to use

4. Materials required but not provided

- Microtiter plate reader fitted with appropriate filters (450 nm required with optional 620 nm 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.

Wash buffer 1X: Once prepared, store at 2-8°C for up to 30 days.

Assay Buffer 1X: Once prepared, store at 2-8°C for up to 30 days.

Reconstituted Standard: Once prepared use immediately and do not store.

Diluted Biotin-conjugate Anti-mIP-10: Once prepared use immediately and do not store.

Diluted Streptavidin-HRP: Once prepared use immediately and do not store.

6. Specimen collection, processing & storage

Cell culture supernatants, 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 Stop Solution and Substrate Solution. 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 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 Stop Solution 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 Substrate Solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the Substrate Solution with metal to prevent colour development. Warning substrate Solution 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 Substrate 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 substrate Solution within 15 min of the washing of the microtiter 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 and zero 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 2-8ºC 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	500	500										
B	250	250										
C	125	125										
D	62.5	62.5										
E	31.3	31.3										
F	15.6	15.6										
G	zero	zero										
H												

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

8.2. Preparation of Wash Buffer

Pour entire contents (50 ml) of the Wash Buffer Concentrate (20x) into a clean 1000 ml graduated cylinder.
Bring to final volume of 1000 ml with glass-distilled or deionized water.
Mix gently to avoid foaming.
Transfer to a clean wash bottle and store at 2° to 25°C.

8.3. Preparation of Assay Buffer

Pour the entire contents (5 ml) of the Assay Buffer Concentrate (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.
Transfer to a clean wash bottle and store at 2° to 8°C.

8.4. Preparation of Standard

Standard vials must be reconstituted with the volume of distilled water shown on the vial immediately prior to use. This reconstitution gives a stock solution of 1000 pg/ml of mIP-10. Mix the reconstituted standard gently by inversion only. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 500 to 15.6 pg/ml. A fresh standard curve should be produced for each new assay.

- Add 100µl of Sample Diluent to all standard and blank wells
- Immediately after reconstitution, add 100 µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 500 pg/ml. Mix the well contents by repeated aspirations Transfer 100 µl 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 100 µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 500 to 15.6 pg/ml.
- Discard 100 µl 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 Biotin Conjugate

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

8.6. Preparation of Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

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 Biotin conjugate (section 8.5) and Streptavidin-HRP (section 8.6) should occur immediately before use.

Assay Step		Details
1.	Wash	Remove the pre-coated plate from the sealed pouch, removed any un-needed strips and wash the plate as follows: a) Dispense 0.3 ml of **1x washing solution** into each well b) Aspirate the contents of each well c) Repeat step a and b
2.	Preparation	**Prepare Standard curve** as shown in section 8.4
3.	Addition	Add 100µl of **Sample Diluent** in duplicate to the blank wells
4.	Addition	Add 50µl of **Sample Diluent** to all sample wells
5.	Addition	Add 50µl of each **sample** in duplicate to appropriate number of wells
6.	Addition	Add 50µl of diluted **biotin-conjugate** to all wells
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **2 hours** on a microplate shaker
8.	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 five times
9.	Addition	Add 100µl of diluted **Streptavidin-HRP** solution into all wells
10.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour** on a microplate shaker
11.	Wash	Repeat wash step 8.
12.	Addition	Add 100µl of ready-to-use **Substrate Solution** into all wells
13.	Incubation	Incubate in the dark for **10 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.
14.	Addition	Add 100µl of **Stop Solution** into all wells
Read the absorbance value of each well (immediately after step 14.) 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*

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

10. Data Analysis

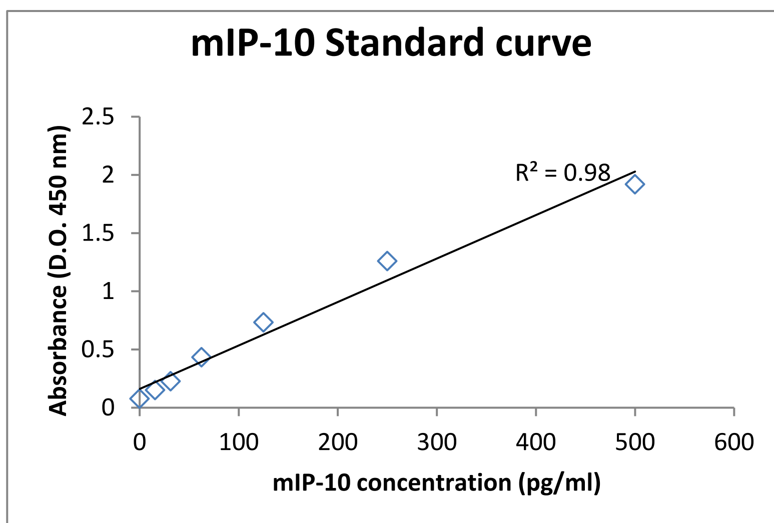
Calculate the average absorbance values for each set of duplicate standards, and samples. Ideally duplicates should be within 20% of the mean.

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

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

Example mIP-10 Standard curve

Standard	mIP-10 Conc	OD (450nm) mean	CV (%)
1	500	1.920	0.7
2	250	1.260	0.3
3	125	0.733	4.9
4	62.5	0.434	4.6
5	31.3	0.228	3.0
6	15.6	0.152	3.4
zero	0	0.077	4.8



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 samples which have been diluted 1:2 according to the protocol, 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 Wash Buffer, fill with Wash 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 limit of detection of murine IP-10 defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 6.5 pg/ml (mean of 6 independent assays).

12.2. Specificity

The assay detects both natural and recombinant murine IP-10.
The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations. There was no cross reactivity detected.

12.3. Precision

Intra-assay
Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 7 serum or plasma samples (spiked or unspiked) containing different concentrations of murine IP-10. 2 standard curves were run on each plate.
The calculated overall intra-assay coefficient of variation was 8.4%.

Inter-assay
Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 7 serum or plasma samples (spiked or unspiked) containing different concentrations of murine IP-10. 2 standard curves were run on each plate.
The calculated overall inter-assay coefficient of variation was 7.2%.

12.4. Dilution Parallelism

Cell culture supernatant samples with different levels of murine IP-10 were analysed at serial 2 fold dilutions with 4 replicates each.
The recovery ranged from 80.3% to 99.2% with an overall recovery of 90.9%.

12.5. Spike Recovery

The spike recovery was evaluated by spiking 3 levels of mouse IP-10 into cell culture supernatant. Recoveries were determined in 3 independent experiments with 6 replicates each.
The unspiked cell culture supernatant was used as blank in these experiments.
The recovery ranged from 55% to 118% with an overall mean recovery of 90%.

12.6. Stability

Storage Stability
Aliquots of cell culture supernatant samples were stored at -20°C, 2-8°C and room temperature (RT), and the murine IP-10 level determined after 24 h. There was no significant loss of murine IP-10 immunoreactivity detected during storage under above conditions.

Freeze-thaw Stability
Aliquots of cell culture supernatant samples were stored at -20°C and thawed 5 times, and the murine IP-10 levels determined. There was no significant loss of murine IP-10 immunoreactivity detected by freezing and thawing.

12.7. Expected serum values

A panel of sera and plasma samples from randomly selected mice was tested for mouse IP-10.
The detected murine IP-10 levels ranged between 0 and 39 pg/ml with a mean level of 16.4 pg/ml for sera samples. Mean levels of plasma samples were of 16.0 pg/ml (EDTA) and 14.0 pg/ml (citrate).

13. Assay Summary

Total procedure length: 3h10min

Add sample, standard and 50µl Biotin-conjugate

Incubate 2 hours at room temperature

Wash six times

Add 100µl of Streptavidin-HRP

Incubate 1 hour at room temperature

Wash six times

Add 100µl of Substrate Solution
Protect from light. Let the color develop for 10 min.

Add 100µl Stop Solution

Read Absorbance at 450 nm

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