PRODUCT INFORMATION

MCP-3 (Human) ELISA

Enzyme-linked immunosorbent assay for

detection of human MCP-3.

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

REF ***IB49666***

Manufactured for:
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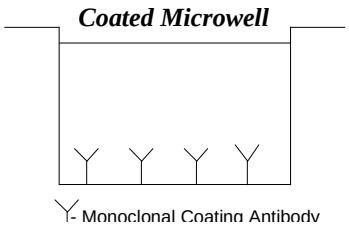
1. Intended Use

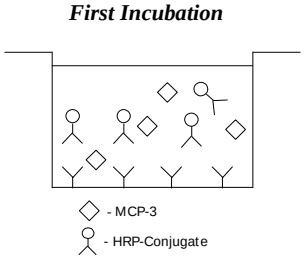
The MCP-3 ELISA is an enzyme-linked immunosorbent assay for the detection of monocyte chemoattractant protein-3 levels in cell culture supernatants, human serum, plasma, amniotic fluid, or other body fluids. For research use only, not for use in diagnostic procedures.

2. Principle of the Test

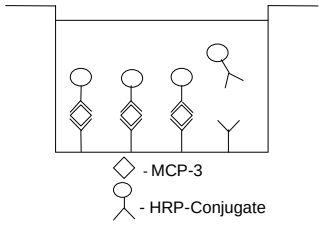
An anti-MCP-3 monoclonal coating antibody is adsorbed onto microwells.



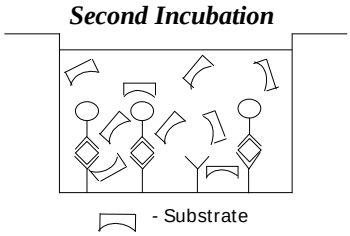
MCP-3 present in the unknown or calibrator binds to antibodies adsorbed to the microwells; a HRP-conjugated monoclonal anti-MCP-3 antibody is added and binds to MCP-3 captured by the first antibody.

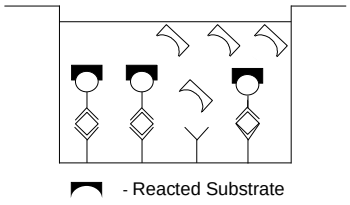


Following incubation unbound enzyme conjugated anti-MCP-3 is removed during a wash step and substrate solution reactive with HRP is added to the wells.



A coloured product is formed in proportion to the amount of MCP-3 present in the unknown. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A calibrator curve is prepared from seven MCP-3 calibrator dilutions and MCP-3 unknown concentration determined.





~~3.~~2. Reagents Provided

1 aluminium pouch with **a Microwell Plate coated with Monoclonal Antibody** (murine) to human MCP-3

1 vial (0.1 ml) **HRP-Conjugate** anti-MCP-3 monoclonal (murine) antibody

2 vials **MCP-3 Calibrator**, lyophilized, 2000pg/ml upon reconstitution

1 bottle (50 ml) **Wash Buffer Concentrate 20x** (PBS with 1% Tween 20);

1 vial (5 ml) **Assay Buffer Concentrate 20x** (PBS with 1% Tween 20 and 10% BSA);

1 vial (12 ml) **Sample diluent** (protein matrix)

1 vial (7 ml) **Substrate Solution I** (tetramethyl-benzidine).

1 vial (7 ml) **Substrate Solution II** (0.02 % buffered hydrogen peroxide).

1 vial (12 ml) **Stop Solution** (1M Phosphoric acid)

1 vial (0.4 ml) **Blue-Dye**

1 vial (0.4 ml) **Green-Dye**

2 adhesive **Plate Covers**

Reagent Labels

~~4.~~2. Storage Instructions – ELISA Kit

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on 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.

~~5.~~2. Collection and Handling of Unknowns

Cell culture supernatants, human serum, EDTA, or heparinized plasma, amniotic fluid, or other body fluids are suitable for use in the assay. Remove the serum or plasma from the clot or red cells, respectively, as soon as possible after clotting and separation.

Unknowns containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic unknowns.

Unknowns must be stored frozen at -20°C to avoid loss of bioactive MCP-3. If unknowns are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles. Prior to assay, frozen sera or plasma should be brought to room temperature slowly and mixed gently and properly diluted in the microwells with Sample Diluent.

~~6.~~2. Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 20 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microplate reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform linear regression analysis.

~~7.~~2. Precautions for Use

- - All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or unknowns are handled.
- Avoid contact of skin or mucous membranes with kit reagents or unknowns.
- Rubber or disposable latex gloves should be worn while handling kit reagents or unknowns.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or unknowns which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acid inactivates the conjugate.

- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose unknowns and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

~~8.~~2. Preparation of Reagents

Except for the HRP-Conjugate (reagent C.) and the TMB Substrate Solution (reagent E.) the reagents should be prepared before starting the test procedure.

8.1 Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved. Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4. Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer is stable for 30 days. Wash Buffer may be prepared as needed according to the following table.

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

8.2 Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer Concentrate** (5.0 ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days. Assay Buffer may be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

8.3 Preparation of HRP-Conjugate

The **HRP-Conjugate** must be diluted 1:100 with Assay Buffer (reagent B.) just prior to use in a clean plastic test tube. Please note that the HRP-Conjugate should be used within 30 minutes after dilution. HRP-Conjugate may be prepared as needed according to the following table:

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

8.4 Preparation of MCP-3 Calibrator

Reconstitute **MCP-3 Calibrator** by addition of distilled water. Reconstitution volume is stated on the label of the calibrator vial. Swirl or mix gently to insure complete and homogeneous solubilization.

8.5 TMB Substrate Solution

Using clean pipettes and containers known to be metal free, dispense an equal volume of **Substrate Solution I** into **Substrate Solution II** and swirl gently to mix. The TMB Substrate Solution may develop a yellow tinge over time. This does not seem to affect product performance. A blue colour present in the TMB Substrate Solution, however, indicates that it has been contaminated and must be discarded. The TMB Substrate Solution must be used within a few minutes after mixing. Warm to room temperature before use. Avoid direct exposure of TMB reagents to intense light and oxidizing agents during storage or incubation.

Substrate preparation by assay size:

Number of Strips	Substrate Solution I (ml)	Substrate Solution II (ml)
1 - 6	3.0	3.0
1- 12	6.0	6.0

8.6 Addition of colour-giving reagents: Blue-Dye, Green-Dye

In order to help our customers to avoid any mistakes in pipetting the IBL-America ELISAs, IBL-America offers a tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure. This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, the dye solutions from the stocks provided (***Blue-Dye, Green-Dye***) can be added to the reagents according to the following guidelines:

1. Diluent: Before dilution of unknowns add the ***Blue-Dye*** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of ***Blue-Dye***, proceed according to the instruction booklet.

5 ml Sample Diluent	20 µl ***Blue-Dye***
12 ml Sample Diluent	48 µl ***Blue-Dye***

2. HRP-Conjugate: Before dilution of the concentrated conjugate, add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the Assay Buffer used for the final conjugate dilution. Proceed after addition of ***Green-Dye*** according to the instruction booklet, preparation of HRP-conjugate.

3 ml Assay Buffer	30 µl ***Green-Dye***
6 ml Assay Buffer	60 µl ***Green-Dye***
12 ml Assay Buffer	120 µl ***Green-Dye***

~~9.~~2. Test Protocol

1. Mix all reagents thoroughly without foaming before use.

2. Determine the number of Microwell Strips required to test the desired number of unknowns plus appropriate number of wells needed for running blanks and calibrators. Each unknown, calibrator, blank, and optional control should be assayed in duplicate. Remove extra microwell strips (murine) to human MCP-3 from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

3. Wash the microwell strips twice with approximately 300 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells. After the last wash, tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Do not allow wells to dry.

4. Add 100 µl of **Sample Diluent** in duplicate to all calibrator wells. Prepare calibrator dilutions by pipetting 100 µl of **MCP-3 Calibrator**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix and transfer 100µl to wells B1 and B2 respectively. Take care not to scratch the inner surface of the microwells. Mix the contents of well B1 and B2 and transfer 100 µl to well C1 and C2 respectively. Continue this procedure four times, creating two rows of MCP-3 Calibrator dilutions ranging from 1000-16 pg/ml. Discard 100 µl of the contents from the last microwell used (G1, G2).

Figure 1. Preparation of MCP-3 calibrator dilutions:

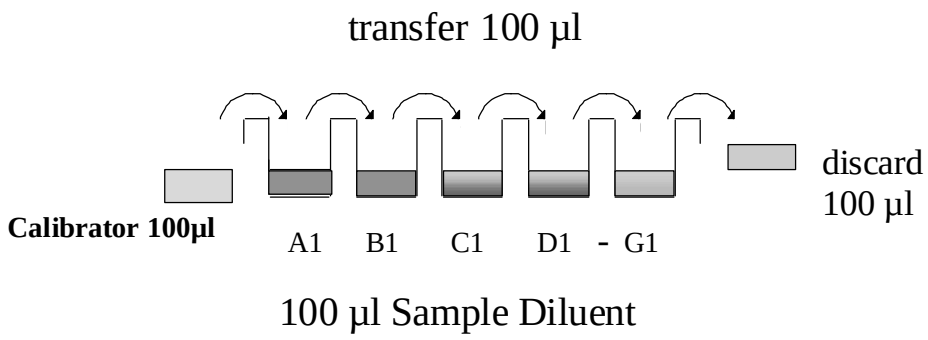


Figure 2. Diagram depicting an example of the arrangement of blanks, calibrators and unknowns in the microwell strips:

	1	2	3	4
A	Calibrator 1 (1000 pg/ml)	Calibrator 1 (1000 pg/ml)	Unknown 1	Unknown 1
B	Calibrator 2 (500 pg/ml)	Calibrator 2 (500 pg/ml)	Unknown 2	Unknown 2
C	Calibrator 3 (250 pg/ml)	Calibrator 3 (250 pg/ml)	Unknown 3	Unknown 3
D	Calibrator 4 (125 pg/ml)	Calibrator 4 (125 pg/ml)	Unknown 4	Unknown 4
E	Calibrator 5 (63 pg/ml)	Calibrator 5 (63 pg/ml)	Unknown 5	Unknown 5
F	Calibrator 6 (32 pg/ml)	Calibrator 6 (32 pg/ml)	Unknown 6	Unknown 6
G	Calibrator 7 (16 pg/ml)	Calibrator 7 (16 pg/ml)	Unknown 7	Unknown 7
H	Blank	Blank	Unknown 8	Unknown 8

~~e.~~5. Add 100 µl of **Sample Diluent** in duplicate to the blank wells.

~~f.~~6. Add 50 µl of **Sample Diluent** to all wells designated for unknowns.

7. Add 50 µl of each **unknown**, in duplicate, to the designated wells and mix the contents.

8. Prepare **HRP-Conjugate**. (Refer to preparation of reagents 8.3)

9. Add 50 µl of diluted (1:100) **HRP-Conjugate** to all wells, including the blank wells.

10. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 1 hour, if available on a rotator set at 100 rpm.

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11. Prepare **TMB Substrate Solution** a few minutes prior to use. (Refer to preparation of reagents 8.5).

12. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point 3. of the test protocol. Proceed immediately to the next step.

13. Pipette 100 µl of mixed **TMB Substrate Solution** to all wells, including the blank wells.

14. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 minutes, if available on a rotator set at 100 rpm. Avoid direct exposure to intense light.

    The colour development on the plate should be monitored and the substrate reaction stopped (see point 15. of this protocol) before positive wells are no longer properly recordable.

    It is recommended to add the stop solution when the highest calibrator has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as an OD of 0.6 – 0.65 is reached.

15. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

16. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the unknowns and the MCP-3 calibrators.

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.~~2. Results

- Calculate the average absorbance values for each set of duplicate calibrators and unknowns. Duplicates should be within 20 percent of the mean.

- Create a calibrator curve by plotting the mean absorbance for each calibrator concentration on the ordinate against the MCP-3 concentration on the abscissa. Draw a best fit curve through the points of the graph.

- To determine the concentration of circulating MCP-3 for each unknown, first find the mean absorbance value on the ordinate and extend a horizontal line to the calibrator curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding MCP-3 concentration.

- **For unknowns which have been diluted according to the instructions given in this manual 1:2, the concentration read from the calibrator curve must be multiplied by the dilution factor (x 2).**

- It is suggested that each testing facility establishes a control of known MCP-3 concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.

Note: Calculation of unknowns with an O.D. exceeding 2.0 may result in incorrect low MCP-3 levels. Such unknowns require further dilution e.g. 1:4, 1:8 with Assay Buffer in order to precisely determine the actual MCP-3 level.

~~11.~~2. Limitations

- Since exact conditions may vary from assay to assay, a calibration curve must be established for every run.
- Bacterial or fungal contamination of either screen unknowns or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure may result in inaccurate results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- Human anti-mouse IgG antibodies (HAMA) may interfere with assays utilizing murine monoclonal antibodies leading to inaccurate results. Serum unknowns containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the unknown.

~~12.~~2. Performance Characteristics

12.1 Spike Recovery

The spike recovery was evaluated by spiking four concentrations of recombinant MCP-3 into human serum. Recoveries were determined in three independent experiments with 6 replicates each. The unspiked serum was used as blank in these experiments. Average recovery ranged from 100% to 113% with an overall mean recovery of 105%.

12.2 Dilution Parallelism

Four serums with different levels of MCP-3 were analyzed at serial two fold dilutions with 4 replicates each. In the table below the per-cent recovery of expected values is listed. The recovery ranged between 82 % and 100 % with an overall recovery of 91 %.

		MCP-3 Concentration (pg/ml)		
Serum	Dilution	Expected Value	Observed Value	% Recovery of Exp. Value
1	1:2	--	1430	100*
	1:4	715	674	94
	1:8	358	350	98
	1:16	179	152	85
2	1:2	--	876	100*
	1:4	438	398	91
	1:8	219	206	94
	1:16	110	90	82
3	1:2	--	312	100*
	1:4	156	140	90
	1:8	78	71	92
	1:16	39	36	92
4	1:2	--	246	100*
	1:4	123	112	91
	1:8	62	50	82
	1:16	31	31	100

* by definition

12.3 Stability of Unknowns

12.3.1 Freeze-Thaw Stability

Aliquots of serum (unspiked or spiked) were stored at -20°C and thawed several times, and the MCP-3 levels determined. There was no significant loss of MCP-3 by repeated freezing and thawing.

12.3.2 Storage Stability

Aliquots of serum (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the MCP-3 level determined after 24 h. There was no loss of MCP-3 immunoreactivity during storage under above conditions.

12.4 Specificity

The assay recognizes both natural and recombinant human MCP-3. To define the specificity of this ELISA several proteins were tested for cross reactivity. There was no cross reactivity observed for any of the proteins tested, notably there was no cross reactivity with MCP-1.

~~13.~~2. Ordering Information

This kit is manufactured for Immuno-Biological Laboratories, Inc. (IBL-America). For ordering information, please contact:

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