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New Substrate Solution

rat MCP-1

Enzyme Immunometric Assay Kit

Catalog No. 900-077

96 Determination Kit

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Description

Assay Designs' rat MCP-1 Enzyme Immunometric Assay (EIA) kit is a complete kit for the quantitative determination of rat MCP-1 in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to rat MCP-1 immobilized on a microtiter plate to bind the rat MCP-1 in the sample. After a short incubation the excess sample is washed out and a monoclonal antibody to rat MCP-1 labeled with the enzyme Horseradish peroxidase is added. This labeled antibody binds to the rat MCP-1 captured on the plate. After a short incubation the excess labeled antibody is washed out and substrate is added. The substrate reacts with the labeled antibody bound to the rat MCP-1 captured on the plate. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of rat MCP-1 in either standards or samples. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

Introduction

Monocyte chemoattractant protein-1 (MCP-1) was first isolated in 1973 by L. C. Altman *et al*, and further characterized by E.J. Leonard *et al* in 1990[3,4]. MCP-1 is a 76 amino acid basic glycoprotein encoded by a highly conserved single gene at chromosome 17q11.2-q21.1, that belongs to the family of chemotactic cytokines known as C-C, or beta- chemokines[5,6]. MCP-1 is highly chemotatic for monocytes, T lymphocytes, basophils, and NK cells during inflammatory responses, and is an example of the prototypical pro-inflammitory chemokine[7-9]. MCP-1 is synthesized by a wide variety of cell types including monocytes, vascular endothelial cells, smooth muscle cells, glomular mesangial cells, osteoblastic cells, and articular chondrocytes in response to pro-inflammitory cytokines such as IL-1, IL-6, TNF-alpha, and INF-gamma[10,11]. In addition to chemotaxis, MCP-1 regulates adhesion molecule expression and cytokine production in monocytes, and can induce the proliferation of other effector cells of the immune system[12,13]. Overexpression of MCP-1 has been associated with a variety of inflammatory diseases such as rhumatoid arthritis and atherosclerosis, and high levels of MCP-1 have been reported in melanoma and invasive breast cancer[14-17]. In addition, MCP-1 has been implicated as a direct mediator of tumor angiogenesis by promoting endothelial cell chemotaxis and proliferation[18].

Precautions

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1. Stop Solution is a 1 normal (1N) sulfuric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the Horseradish peroxidase conjugate is affected by nucleophiles, such as azide, cyanide and hydroxylamine.
3. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
4. The rat MCP-1 Standard provided, Catalog No. 80-0885, should be handled with care, because of the known and unknown effects of MCP-1.
5. The rat MCP-1 Standard and Labeled Antibody should be stored at -20°C. Do not repeatedly freeze-thaw.

Materials Supplied

1. **rat MCP-1 Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0883**
   A strip microtiter plate coated with rabbit antibody specific to rat MCP-1.
2. **rat MCP-1 Labeled Antibody, 1 vial, Catalog No. 80-0884**
   Mouse antibody to rat MCP-1 conjugated to Horseradish peroxidase.
3. **Assay Buffer, 30 mL, Catalog No. 80-0170**
   Phosphate buffered saline containing proteins and detergents.
4. **Labeled Antibody Diluent, 10 mL, Catalog No. 80-0182**
   Phosphate buffered saline containing proteins and detergents.
5. **Wash Buffer Concentrate, 50 mL, Catalog No. 80-0171**
   Phosphate buffered saline containing detergents.
6. **rat MCP-1 Standard, 1 vial, Catalog No. 80-0885**
   A vial containing 3,200 pg of recombinant rat MCP-1.
7. **TMB Substrate, 15 mL, Catalog No. 80-1342**
   A solution of 3,3',5,5' tetramethyl benzidine (TMB) and hydrogen peroxide. Ready to use.
8. **Stop Solution, 11 mL, Catalog No. 80-0176**
   A 1N solution of sulfuric acid in water. Keep tightly capped. Caution: **Caustic.**
9. **rat MCP-1 Assay Layout Sheet, 1 each, Catalog No. 30-0155**
10. **Plate Sealer, 2 each, Catalog No. 30-0012**

Storage

All components of this kit, **except the Labeled Antibody and Standard**, are stable at 4°C until the kit's expiration date. The Labeled Antibody and Standard **must** be stored at -20°C.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 100 µL and 1,000 µL.
3. Disposable test tubes for dilution of samples and standards.
4. Repeater pipet for dispensing 100 µL.
5. Disposable beakers for diluting Wash Buffer.
6. Graduated cylinders.
7. A 37°C incubator.
8. Adsorbent paper for blotting.
9. Microplate reader capable of reading at 450 nm., preferably with correction between 570 nm and 590 nm.
10. Graph paper for plotting the standard curve.

Sample Handling

Assay Designs' EIA is compatible with rat MCP-1 samples in tissue culture medium and serum. Samples diluted sufficiently into Assay Buffer can be read directly from the standard curve.

Culture fluids and serum are suitable for use in the assay. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens. Samples in the majority of tissue culture media, including fetal bovine serum, can be read in the assay if diluted into Assay Buffer. Please refer to page 11 for recommended sample dilutions. Users should only use standard curves generated in Assay Buffer to calculate concentrations of rat MCP-1.

Samples must be stored frozen to avoid loss of bioactive rat MCP-1. If samples are to be run within 24 hours, they may be stored at 4°C. Otherwise, samples must be stored frozen at -70°C to avoid loss of bioactive rat MCP-1. Excessive freeze/thaw cycles should be avoided. Prior to assay, frozen sera should be brought to room temperature slowly and gently mixed by hand. Do not thaw samples in a 37°C incubator. Do not vortex or sharply agitate samples.

Procedural Notes

1. Do not mix reagents from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses plates with removable strips. Unused strips must be kept desiccated at 4ºC in the sealed foil bag. The strips should be used in the frame provided.
8. **Prior to addition of antibody or substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. Wash Buffer

Prepare Wash Buffer by diluting 25 mL of the supplied concentrate with 975 mL of deionized water. This can be stored at 4°C until the kit expiration date, or for 3 months, whichever is earlier.

2. rat MCP-1 Standards

Add 1 mL of deionized water to the rat MCP-1 Standard. Let it sit at room temperature for 5 minutes. Mix it gently. This solution contains 3,200 pg/mL rat MCP-1.

Label six 12 x 75 mm glass tubes #1 through 6. Pipet 220 µL of Assay Buffer into tubes #1 through #6. Add 220 µL of the 3,200 pg/mL standard to tube #1. Vortex. Add 220 µL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #6.

The concentration of rat MCP-1 in tubes #1 through #6 will be 1,600, 800, 400, 200, 100 and 50 pg/mL respectively. See rat MCP-1 Assay Layout Sheet for dilution details.

3. Preparation of Labeled Antibody Conjugate

Add the entire contents of one (1) bottle of Labeled Antibody Diluent to the vial of MCP-1 Antibody Conjugate. Let it stand at room temperature for 5 minutes and then vortex it gently. After reconstitution, any unused Labeled Antibody should be aliquoted and stored at -20°C.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the foil pouch and seal the ziploc. Store unused wells at 4°C.

2. Pipet 100 µL of Assay Buffer into the S0 (0 pg/mL Standard) wells.

3. Pipet 100 µL of Standards #1 through #6 into the appropriate wells.

4. Pipet 100 µL of the Samples into the appropriate wells.

5. Tap the plate gently to mix the contents.

6. Seal the plate and incubate at 37°C for 1 hour.

7. Empty the contents of the wells & wash by adding 400 µL of wash solution to every well. Repeat the wash 6 more times for a total of **7 washes**. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

8. Pipet 100 µL of the Labeled Antibody into each well, except the Blank.

9. Seal the plate and incubate at 37°C for 30 minutes. Prepare Substrate (See page 5, Section 4).

10. Empty the contents of the wells & wash by adding 400 µL of wash solution to every well. Repeat the wash 8 more times for a total of **9 washes**. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

11. Add 100 µL of the Substrate Solution to each well.

12. Incubate for 30 minutes at room temperature in the dark.

13. Add 100 µL of Stop Solution to each well.

14. Blank the plate reader against the Blank wells, read the optical density at 450 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the blank wells from all readings.

Calculation of Results

Several options are available for the calculation of the concentration of rat MCP-1 in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of rat MCP-1 can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

2. Plot the Average Net OD for each standard versus rat MCP-1 concentration in each standard.

3. Plot the Average OD for each sample and extrapolate rat MCP-1 concentration from the graph.

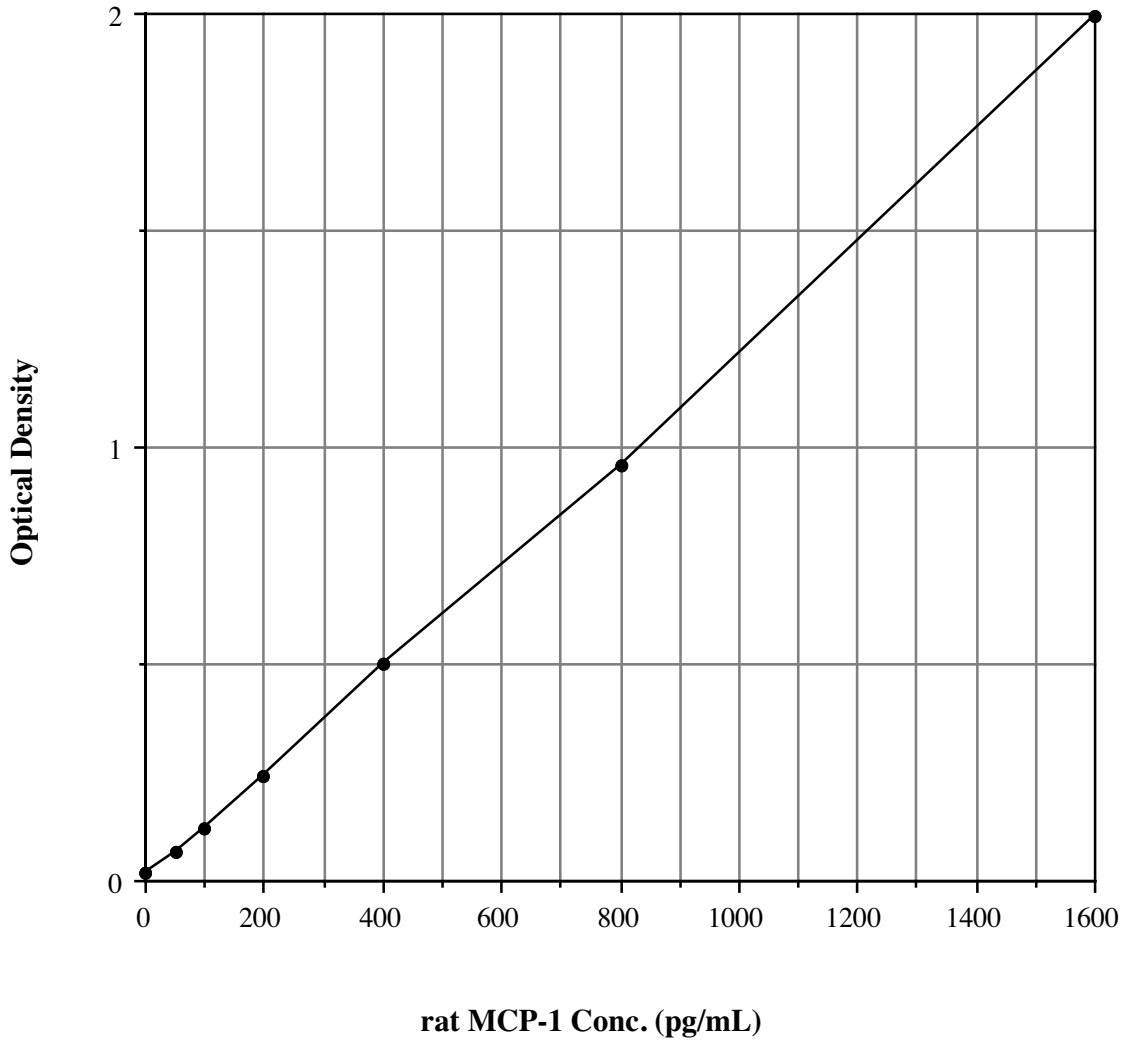
Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

Sample	Average OD	Net OD	**rat MCP-1 (pg/mL)**
Blank	0.048		
0 standard	0.066	0.018	**0**
S1	2.040	1.992	**1,600**
S2	1.007	0.959	**800**
S3	0.545	0.497	**400**
S4	0.286	0.238	**200**
S5	0.169	0.121	**100**
S6	0.113	0.065	**50**

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate rat MCP-1 concentrations; each user must run a standard curve for each assay.



Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[19].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for twelve (12) wells run at 0 pg/mL rat MCP-1, and comparing to the average optical density for twelve (12) wells run with Standard #6. The detection limit was determined as the concentration of rat MCP-1 measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Average Optical Density for the 0 pg/mL Standard =	0.047 ± 0.009 (19.5%)
Average Optical Density for Standard #6 =	0.091 ± 0.006 (6.8%)
Delta Optical Density (50-0 pg/mL) =	0.044
2 SD's of the 0 pg/mL Standard = 2 x 0.009 =	0.018

Sensitivity = $\frac{0.018}{0.044} \times 50 \text{ pg/mL} =$ **20.45 pg/mL**

Linearity

A sample containing 2,125 pg/mL rat MCP-1 was diluted 5 times 1:2 into Assay Buffer and measured in the assay. The data was plotted graphically as actual rat MCP-1 concentration versus measured rat MCP-1 concentration.

The line obtained had a slope of 0.8746 and a correlation coefficient of 0.9987.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of rat MCP-1 and running these samples multiple times (n=24) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of rat MCP-1 in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of rat MCP-1 determined in these assays as calculated by a curve fitting program.

	rat MCP-1 (pg/mL)	Intra-assay %CV	Inter-assay %CV
Low	82.9	5.2	
Medium	449.3	5.9	
High	1,767.2	4.6	
Low	83.6		3.5
Medium	455.3		3.6
High	1,919.2		4.5

Cross Reactivities

The cross reactivities for a number of related compounds was determined by dissolving the cross reactant in Assay Buffer. These samples were then measured in the rat MCP-1 assay, and the measured rat MCP-1 concentration calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	Cross Reactivity
Rat MCP-1	100%
Rat GRO/CINC-1	<0.1%
Rat GRO/CINC-2α	<0.1%
Rat GRO/CINC-2β	<0.1%
Rat GRO/CINC-3	<0.1%
Rat Rantes	<0.1%
Rat MIP-1α	<0.1%
Rat IL-1β	<0.1%

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

Rat MCP-1 concentrations were measured in tissue culture media (10% FBS added RPMI-1640) and rat serum. Rat MCP-1 was spiked into the undiluted samples of these media which were then diluted with the kit Assay Buffer and assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Tissue Culture Media	99.6	None
rat Serum	93.6	1:8 - 1:128

* See Sample Handling instructions on page 4 for details.

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Material Safety Data Sheet (MSDS) available on our website or by fax.

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