Quantikine® ELISA

Human CCL20/MIP-3α Immunoassay

Catalog Number DM3A00

For the quantitative determination of human Macrophage Inflammatory Protein 3 alpha (MIP-3α) concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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MANUFACTURED AND DISTRIBUTED BY:

USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

MIP-3α [also known as LARC (liver and activation-regulated chemokine), Exodus-1 or CCL20] is a CC chemokine with a selective chemotactic activity for lymphocytes and dendritic cells (DCs). It was independently cloned by three separate groups in 1997 from the GenBank human expressed sequence tag (EST) database (1-3). MIP-3α plays an important role in the regulation of DC trafficking, and recruitment and activation of T cells (for reviews, see references 4 and 5).

The full-length cDNA for MIP-3α encodes a protein of 96 amino acids (aa) that shows relatively low sequence similarity to other human CC chemokines. It shares the highest aa sequence homology with MIP-1β (28% identity) (2). Human MIP-3α demonstrates 64% sequence identity with the mouse orthologue of MIP-3α (6). MIP-3α contains the four conserved cysteine residues as well as three out of five other aa residues that are highly conserved among CC chemokines. The tyrosine and threonine residues that are typically present between the second and third cysteine residues in other CC chemokines are substituted in MIP-3α with phenylalanine and alanine, respectively (2). MIP-3α also differs from other CC chemokines in that it maps to human chromosome 2 vs. chromosome 17.

The G protein-coupled chemokine receptor CCR6, has been identified as a functional receptor for MIP-3α (7-10). Activation of CCR6 leads to pertussis toxin-sensitive and phospholipase C-dependent intracellular Ca^{2+} mobilization (7, 8). It is expressed on memory T cells, B cells and DCs (11). Cytokines can influence the expression of CCR6. TGF-β1, for example, can up-regulate expression of CCR6 on DCs (12, 13), whereas TNF-α and IL-4 can both decrease CCR6 protein levels (14). Cell surface expression of CCR6 determines responsiveness to MIP-3α. B cell responsiveness to MIP-3α and CCR6 expression, however, is acquired only following emigration from the bone marrow to the periphery and during maturation (15).

MIP-3α is produced by activated cells, including monocytes, T cells, endothelial cells, epithelial cells, and fibroblasts and is expressed in liver, lung, and some lymphoid tissues. The expression of MIP-3α mRNA is predominant in inflamed and mucosal tissues (7). Expression of the MIP-3α protein can be induced by cytokines such as TNF-α, IL-1β, CD40 Ligand, IFN-γ, and IL-17 (16). Phorbol myristate acetate can also induce MIP-3α expression in several human cell lines (2).

MIP-3α may contribute to the pathobiology of several disease states via autocrine and/or paracrine mechanisms. Cell surface expression of MIP-3α and CCR6 is markedly up-regulated in psoriasis, strongly suggesting that the interaction between MIP-3α and its receptor is important for T cell recruitment to lesional psoriatic skin (16). Pancreatic cancer cell lines over-express MIP-3α in comparison to normal pancreatic cells (17). Thyroid papillary carcinoma cells release HGF, which in turn induces expression of MIP-3α (18). DCs are then recruited in response to MIP-3α and subsequently infiltrate the tumor mass.

Like MIP-3α, defensins are also chemotactic for immature DCs and memory T cells. They may contribute to host defense by disrupting the cytoplasmic membranes of microorganisms. Beta-defensin may competitively displace binding of MIP-3α to CCR6 thus promoting adaptive immune responses by recruiting DCs and T cells to sites of microbial invasion (19).

The Quantikine Human MIP-3α Immunoassay is a 4.5 hour solid phase ELISA designed to measure MIP-3α levels in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant human MIP-3α and antibodies raised against the recombinant protein. Results obtained for naturally occurring human MIP-3α showed linear curves that were parallel to the standard curves obtained using the kit standards. These results indicate that this kit can be used to determine relative mass values for natural human MIP-3α.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for MIP-3α has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any MIP-3α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for MIP-3α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of MIP-3α bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- It is important that the Calibrator Diluent selected for the standard curve be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

<table>
<tr><th>PART</th><th>PART #</th><th>DESCRIPTION</th><th>STORAGE OF OPENED/
RECONSTITUTED MATERIAL</th></tr>
<tr><td>MIP-3α Microplate</td><td>890831</td><td>96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against MIP-3α.</td><td>Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.*</td></tr>
<tr><td>MIP-3α Standard</td><td>890833</td><td>Recombinant human MIP-3α in a buffered protein base with preservatives; lyophilized. Refer to vial label for reconstitution volume.</td><td>Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>MIP-3α Conjugate</td><td>890832</td><td>21 mL of polyclonal antibody against MIP-3α conjugated to horseradish peroxidase with preservatives.</td><td rowspan="8">May be stored for up to 1 month at 2-8 °C.*</td></tr>
<tr><td>Assay Diluent RD1-57</td><td>895207</td><td>11 mL of a buffered protein base with blue dye and preservative.</td></tr>
<tr><td>Calibrator Diluent RD6-21</td><td>895261</td><td>21 mL of a buffered protein base with preservatives. For cell culture supernate samples.</td></tr>
<tr><td>Calibrator Diluent RD6U</td><td>895148</td><td>21 mL of animal serum with preservatives. For serum/plasma samples. May contain a precipitate. Mix well before and during use.</td></tr>
<tr><td>Wash Buffer Concentrate</td><td>895003</td><td>21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.</td></tr>
<tr><td>Color Reagent A</td><td>895000</td><td>12 mL of stabilized hydrogen peroxide.</td></tr>
<tr><td>Color Reagent B</td><td>895001</td><td>12 mL of stabilized chromogen (tetramethylbenzidine).</td></tr>
<tr><td>Stop Solution</td><td>895032</td><td>6 mL of 2 N sulfuric acid.</td></tr>
<tr><td>Plate Sealers</td><td>N/A</td><td colspan="2">4 adhesive strips.</td></tr>
</table>

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Test tubes for dilution of standards.
- Human MIP-3α Controls (optional; available from R&D Systems, Inc.).

PRECAUTIONS

Calibrator Diluent RD6U contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Hemolyzed or lipemic samples are not suitable for use in this assay.

REAGENT PREPARATION

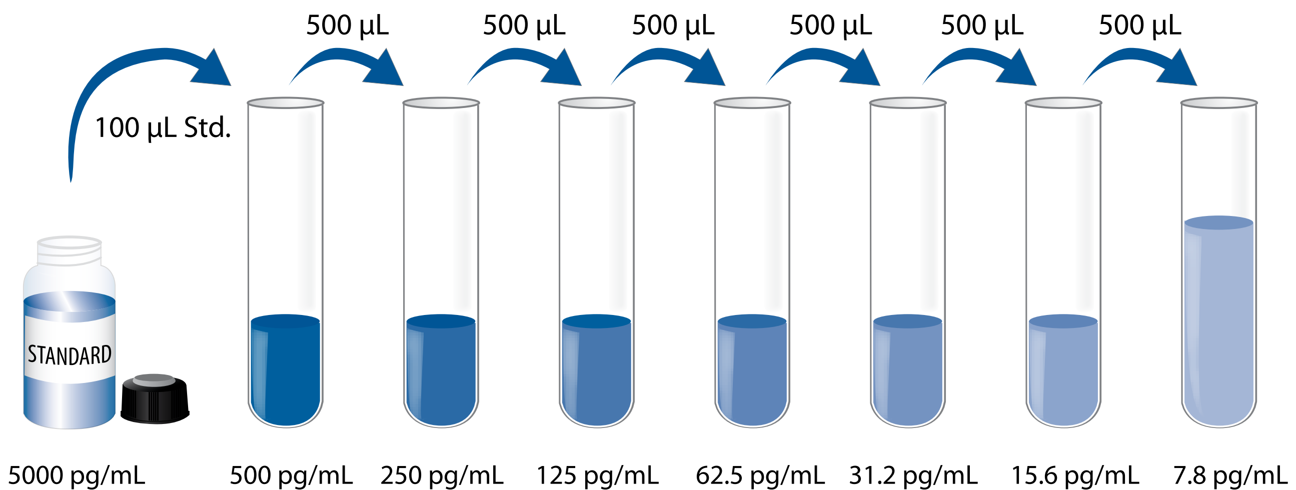
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well.

MIP-3α Standard - **Refer to vial label for standard reconstitution volume.** Reconstitute the MIP-3α Standard with deionized or distilled water. This reconstitution produces a stock solution of 5000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 μL of Calibrator Diluent RD6-21 (*for cell culture supernate samples*) or Calibrator Diluent RD6U (*for serum/plasma samples*) into the 500 pg/mL tube. *Calibrator Diluent RD6U may contain a precipitate. Mix well before and during use.* Pipette 500 μL of the appropriate Calibrator Diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 500 pg/mL dilution serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1-57 to each well.
4. Add 100 µL of Standard, sample, or control per well. Cover with the adhesive strip provided and incubate for 2 hours at room temperature. A plate layout is provided to record the standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper toweling.
6. Add 200 µL of MIP-3α Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

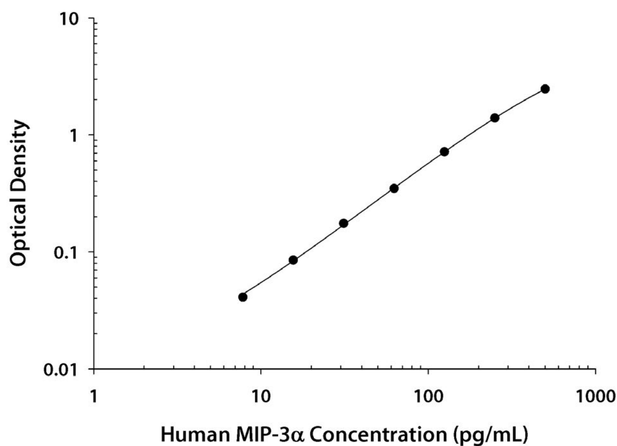
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, contruct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the MIP-3α concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

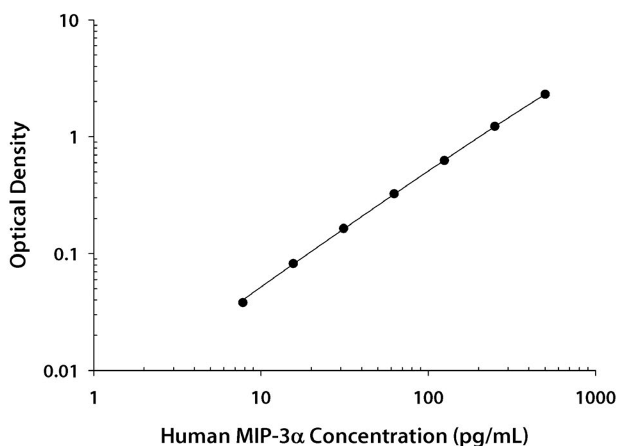
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CALIBRATOR DILUENT RD6-21



(pg/mL)	O.D.	Average	Corrected
0	0.012 0.012	0.012	—
7.8	0.055 0.051	0.053	0.041
15.6	0.097 0.096	0.097	0.085
31.2	0.193 0.181	0.187	0.175
62.5	0.375 0.345	0.360	0.348
125	0.750 0.708	0.729	0.717
250	1.427 1.378	1.403	1.391
500	2.524 2.428	2.476	2.464

CALIBRATOR DILUENT RD6U



(pg/mL)	O.D.	Average	Corrected
0	0.015 0.013	0.014	—
7.8	0.051 0.052	0.052	0.038
15.6	0.095 0.097	0.096	0.082
31.2	0.182 0.174	0.178	0.164
62.5	0.344 0.333	0.339	0.325
125	0.673 0.607	0.640	0.626
250	1.247 1.226	1.237	1.223
500	2.322 2.315	2.319	2.305

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	66.2	180	372	68.7	186	357
Standard deviation	1.84	3.83	15.1	4.51	13.0	28.8
CV (%)	2.8	2.1	4.1	6.6	6.9	8.1

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	55.9	161	323	72.5	200	386
Standard deviation	1.62	4.28	9.10	6.05	17.9	35.3
CV (%)	2.9	2.7	2.8	8.3	8.9	9.1

RECOVERY

The recovery of MIP-3α spiked to three different levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=8)	101	88-115%
Serum (n=5)	103	94-112%
EDTA plasma (n-5)	104	97-115%
Heparin plasma (n=5)	104	98-112%

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LINEARITY

To assess linearity of the assay, samples containing and/or spiked with high concentrations of MIP-3α in various matrices were diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=5)	Serum (n=6)	EDTA plasma (n=5)	Heparin plasma (n=5)
1:2	Average % of Expected	99	104	102	100
	Range (%)	94-101	98-107	100-105	97-104
1:4	Average % of Expected	102	102	103	99
	Range (%)	91-108	98-106	99-110	96-105
1:8	Average % of Expected	98	100	102	98
	Range (%)	85-104	99-106	99-106	95-106
1:16	Average % of Expected	97	98	100	97
	Range (%)	89-110	90-101	95-107	92-105

SENSITIVITY

Thirty-five assays were evaluated and the minimum detectable dose (MDD) ranged from 0.10-0.87 pg/mL. The mean MDD was 0.47 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human MIP-3α produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of MIP-3α in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=74)	26.3	22	ND-143
EDTA plasma (n=34)	14.3	41	ND-42.3
Heparin plasma (n=34)	14.2	59	ND-47.2

ND=Non-detectable

Cell Culture Supernates -

Human peripheral blood mononuclear cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 5% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernate were removed and assayed for levels of natural MIP-3α.

Sample Type	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated	182.5	321.5
Stimulated	2430	660.4

Human endothelial cells (1 x 10^6 cells/mL) were cultured in EGM supplemented with 2% fetal bovine serum, 10 ng/mL hEGF, 1.0 µg/mL hydrocortisone, 50 µg/mL gentamicin, 50 ng/mL amphoteracin B, and 3 mg/mL BBE (bovine brain extract). The cell culture supernate was assayed and measured less than the lowest MIP-3α standard, 7.8 pg/mL.

THP-1 human acute monocytic leukemia cells were cultured in RPMI supplemented with 10% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were culture stimulated with 1 µg/mL IFN-γ and 1 µg/mL LPS. The cell culture supernate was assayed and measured less than the lowest MIP-3α standard, 7.8 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human MIP-3α.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range MIP-3α control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

6Ckine
GROα
GROβ
GROγ
HCC-4
MCP-1
MIP-1α
MIP-1β
MIP-1γ
MIP-3β
PARC
PTN
RANTES

Recombinant mouse:

6Ckine
MIP-3α
MIP-3β

Recombinant rat:

MIP-3α

Recombinant viral:

MIP-I
MIP-II

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

NOTES

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