Quantikine® ELISA

Human MIA Immunoassay

Catalog Number DMIA00

For the quantitative determination of human Melanoma Inhibiting Activity (MIA) concentrations in cell culture supernates, serum, plasma, and saliva.

Note: The reconstitution method has changed. Read this package insert in its entirety before using this product.

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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INTRODUCTION

Melanoma Inhibiting Activity (MIA), also known as cartilage-derived retinoic acid-sensitive protein (CD-RAP), is an approximately 11-15 kDa protein that is expressed as a noncovalent homodimer. MIA is structurally related to OTOR/Otoraplin and MIA-2 in a small family of secreted proteins with one SH3 domain (1-3). SH3 domains are commonly found in cytoplasmic proteins and interact with phosphorylated tyrosine residues in signaling molecules. Mature human MIA shares 90% and 92% amino acid sequence identity with mouse and rat MIA, respectively. MIA is widely expressed in developing and regenerating cartilage and in endodermal epithelium and parenchyma of developing lungs (4-6). It is upregulated in several cancers including malignant melanoma, lung adenoma, metastatic oral squamous cell carcinoma, neurofibromatosis type 1 (NF-1)-related tumors, and pancreatic cancer (1, 6-10). MIA serum levels are elevated in advanced malignant melanoma and NF1-related tumors (9, 11-14) as well as in rheumatoid arthritis (14). Synovial fluid levels of MIA are elevated in mild osteoarthritis and rheumatoid arthritis (5). They are also elevated in cerebrospinal fluid following spinal cord damage resulting from injury or disease (15).

MIA disrupts cellular interactions with the extracellular matrix by binding to Integrins α4β1 and α5β1 (3, 16). It competes with Fibronectin fragments for Integrin binding and interferes with Integrin signaling (16). MIA is selectively secreted and internalized from the trailing pole of migrating cells (17, 18). This polarization reduces cellular attachment to the matrix at the trailing pole and contributes to directional tumor cell migration. MIA inhibits tumor cell proliferation and cell-cell contact while enhancing migration and invasion (1, 10, 19-21). Its dimerization is important for the effects on invasion and *in vivo* metastasis (3). In oral squamous cell carcinoma, MIA expression is positively associated with VEGF expression and microvascular density (8). MIA functions as a chemoattractant for mesenchymal stem cells and enhances their BMP-2 and TGF-β3 induced differentiation into chondrocytes (22). MIA-deficient mice exhibit delayed chondrocyte differentiation but enhanced chondrocyte proliferation and cartilage repair (23).

The Quantikine® Human MIA Immunoassay is a 4.5 hour solid phase ELISA designed to measure human MIA in cell culture supernates, serum, plasma, and saliva. It contains *E. coli*-expressed recombinant human MIA and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate recombinant human MIA. Results obtained using natural human MIA showed linear curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for natural human MIA.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human MIA has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any MIA present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human MIA 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 MIA 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.
- If samples generate values higher than the highest standard, further dilute the samples with calibrator diluent and repeat the assay.
- Any variation in 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 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.
- 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.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human MIA Microplate	894531	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human MIA.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human MIA Conjugate	894532	21 mL of a polyclonal antibody specific for human MIA conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human MIA Standard	894533	Recombinant human MIA in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1W	895117	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-26 Concentrate	895525	21 mL of a concentrated buffered protein base with preservatives. *Do not dilute. Use undiluted in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* 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.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards and samples.
- Human MIA controls (Optional; R&D Systems®, Catalog # QC121).

PRECAUTIONS

MIA is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

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 at room temperature 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.*

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Saliva values are decreased when a Salivette® or other collection device is used.*

SAMPLE PREPARATION

Serum and plasma samples require a 2-fold dilution. A suggested 2-fold dilution is 70 µL of sample + 70 µL of Calibrator Diluent RD5-26 Concentrate.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

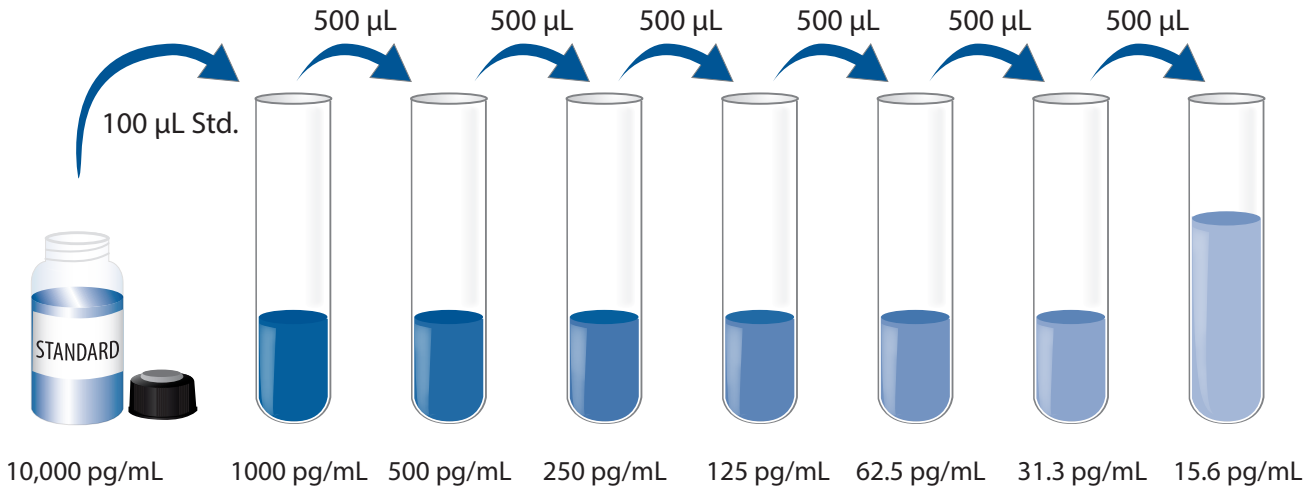
Note: *High concentrations of MIA are found in saliva. Take necessary precautions to protect kit reagents.*

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 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.

Human MIA Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human MIA Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 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 RD5-26 Concentrate into the 1000 pg/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-26 Concentrate 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.

Note: *High concentrations of MIA are found in saliva. Take necessary precautions to protect kit reagents.*

1. Prepare all reagents, working standards, and samples 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 50 µL of Assay Diluent RD1W to each well.
4. Add 50 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 rpm ± 50 rpm.
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 Human MIA Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
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 **on the benchtop. Protect from light.**
9. Add 50 µL of Stop Solution to each well. 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.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

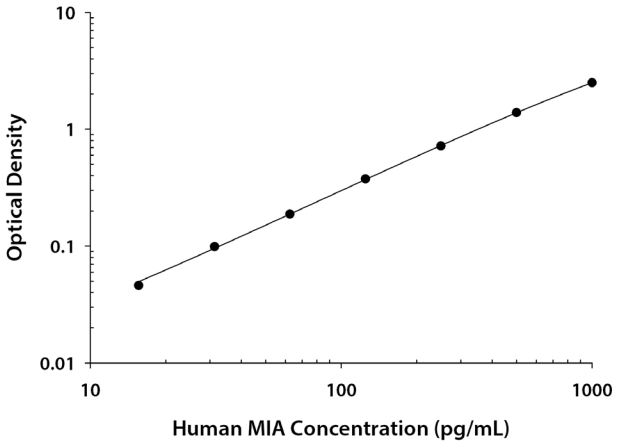
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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, construct 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 human MIA 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

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.041 0.043	0.042	—
15.6	0.088 0.089	0.088	0.046
31.3	0.138 0.144	0.141	0.099
62.5	0.230 0.231	0.230	0.188
125	0.415 0.420	0.417	0.375
250	0.760 0.765	0.762	0.720
500	1.417 1.439	1.428	1.386
1000	2.538 2.545	2.541	2.499

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 twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of kit components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	158	315	622	150	308	627
Standard deviation	5.50	10.9	17.8	7.02	15.7	37.8
CV (%)	3.5	3.5	2.9	4.7	5.1	6.0

RECOVERY

The recovery of human MIA spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	103	101-107%
Serum* (n=4)	101	91-118%
EDTA plasma* (n=4)	98	85-115%
Heparin plasma* (n=4)	99	85-113%
Saliva (n=4)	102	86-110%

*Samples were diluted prior to assay as directed in the Sample Preparation section.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human MIA were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)	Saliva (n=4)
1:2	Average % of Expected	99	100	103	105	100
	Range (%)	98-101	97-103	99-107	104-110	97-105
1:4	Average % of Expected	94	105	106	106	102
	Range (%)	82-100	99-114	99-110	101-110	100-103
1:8	Average % of Expected	97	103	106	107	105
	Range (%)	93-101	96-115	97-117	101-114	103-107
1:16	Average % of Expected	94	91	96	99	109
	Range (%)	92-97	84-109	86-114	84-115	106-110

*Samples were diluted prior to assay.

SENSITIVITY

Ninety-one assays were evaluated and the minimum detectable dose (MDD) of human MIA ranged from 0.580-8.46 pg/mL. The mean MDD was 2.07 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. 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 MIA produced at R&D Systems®.

SAMPLE VALUES

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

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=35)	999	620-1508	225
EDTA plasma (n=35)	1077	652-1584	235
Heparin plasma (n=35)	1088	648-1732	254

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Saliva (n=13)	161	92	ND-513

ND=Non-detectable

Cell Culture Supernates:

HT-29 human colon adenocarcinoma cells (5 x 10^5 cells/100 mL) were cultured in McCoy's Medium 5A supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 7 days. An aliquot of the cell culture supernate was removed, assayed for human MIA, and measured 1040 pg/mL.

Hs 294T human melanoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 2 days. An aliquot of the cell culture supernate was removed, assayed for human MIA, and measured 5900 pg/mL.

Sk-Mel-28 human malignant melanoma cells (2 x 10^5 cells/100 mL) were cultured in MEM-NEAA supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 5 days. An aliquot of the cell culture supernate was removed, assayed for human MIA, and measured 16.1 ng/mL.

A375 human melanoma cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 5 days. An aliquot of the cell culture supernate was removed, assayed for human MIA, and measured 16.3 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human MIA.

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 human MIA control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

BMP-2
Cadherin-7
Fas Ligand
FGF basic
IL-2
IL-5
IL-8
IL-10
IL-13
Integrin α4
Integrin α4β1
Integrin α5β1
Integrin β1
Otoraplin
VEGF

Natural proteins:

human Fibronectin
human TGF-β1

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