Quantikine® ELISA

Mouse MFG-E8 Immunoassay

Catalog Number MFGE80

For the quantitative determination of mouse Milk Fat Globulin E8 (MFG-E8) 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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INTRODUCTION

Milk Fat Globulin Protein E8 (MFG-E8), also known as Lactadherin, MP47, breast epithelial antigen BA46, and SED1, is a 66-75 kDa pleiotropic secreted glycoprotein that promotes mammary gland morphogenesis, angiogenesis, and tumor progression. MFG-E8 also plays an important role in tissue homeostasis and the prevention of inflammation (1). Mature mouse MGF-E8 contains two N-terminal EGF-like domains, a Pro/Thr-rich segment, and two C-terminal F5/8-type discoidin-like domains (2). Mature mouse MFG-E8 shares 63% and 94% amino acid (aa) sequence identity with comparable regions of human and rat MFG-E8, respectively. Alternate splicing of mouse MFG-E8 generates a short isoform lacking the Pro/Thr-rich region which contains sites for O-linked glycosylation and tyrosine sulfation (3, 4). In humans, a 50 aa internal proteolytic fragment of MFG-E8 (known as Medin) is a major component of aortic medial amyloid deposits (5).

MFG-E8 is expressed in the mammary glands of lactating mice, and it is released into the milk in complex with lipid-containing milk fat globules (6, 7). It is also found in multiple other cell types including endothelial cells and smooth muscle cells of the vasculature (8), immature dendritic cells (9, 10), and at the acrosomal cap of testicular and epididymal sperm (11). The short isoform (50-55 kDa) is widely expressed, while the long isoform is preferentially expressed in the lactating mammary gland (3).

An RGD motif within the second EGF-like domain mediates MFG-E8 binding to the Integrins αVβ3 and αVβ5 (8, 12, 13). Integrin αVβ3 associates with VEGF R2 on vascular endothelial cells, and the interaction of MFG-E8 with this Integrin potentiates the angiogenic action of VEGF through VEGF R2 (8, 14). The second discoidin-like domain mediates the binding of MFG-E8 to phosphatidylserine (PS) and other phospholipids (13, 15, 16). MFG-E8 functions as a bridge between PS on apoptotic cells and Integrin αVβ3 on phagocytes, leading to the clearance of apoptotic thymocytes (9, 16). The removal of apoptotic cell debris serves to reduce inflammation and tissue damage in a variety of settings. It reduces the risk of developing autoimmunity by enabling the clearance of apoptotic B cells during the germinal center reaction (17, 18). It reduces inflammation and disease progression in colitis by preventing Osteopontin from binding and activating Integrin αVβ3 (19). MFG-E8 binding to PS on the surface of injured intestinal epithelial cells promotes their migration and the regeneration of epithelial integrity (20). MFG-E8 additionally limits disease progression by promoting the engulfment of apoptotic bodies in atherosclerotic plaques and prion-infected brain by macrophages and microglia, respectively (21, 22). MFG-E8 also promotes the removal of excess Collagen in fibrotic lungs (23), limits gut injury after ischemia-reperfusion (24), and blocks rotavirus infection (25). Its tissue-protective role also impairs anti-tumor immunity and chemotherapy-induced apoptosis (26).

The Quantikine Mouse MFG-E8 Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse MFG-E8 in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant mouse MFG-E8 and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant factor accurately. Results obtained using natural mouse MFG-E8 showed dose-response curves that were parallel to the standard curves obtained using the recombinant kit standards. These results indicate that this kit can be used to determine relative mass values for natural mouse MFG-E8.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse MFG-E8 has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any mouse MFG-E8 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse MFG-E8 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of mouse MFG-E8 bound in the initial step. The sample values are then read off the standard curve.

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 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 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.

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
Mouse MFG-E8 Microplate	894085	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse MFG-E8.	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.*
Mouse MFG-E8 Conjugate	894086	12 mL of a polyclonal antibody specific for mouse MFG-E8 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Mouse MFG-E8 Standard	894087	40 ng of recombinant mouse MFG-E8 in a buffered protein base with preservatives; lyophilized.	
Mouse MFG-E8 Control	894088	Recombinant mouse MFG-E8 in a buffered protein base with preservatives; lyophilized. The concentration range of mouse MFG-E8 after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.	
Assay Diluent RD1-55	895066	11 mL of a buffered protein base with blue dye and preservatives.	
Calibrator Diluent RD5-20 Concentrate	895346	21 mL of a concentrated buffered protein base with preservatives.	
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	895174	23 mL of diluted hydrochloric 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.
- **Polypropylene** test tubes for dilution of standards and samples.

PRECAUTIONS

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

Some components in this kit contain ProClin® 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. Please 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. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *EDTA plasma is not suitable for use in this assay.*
Citrate plasma has not been validated for use in this assay.

SAMPLE PREPARATION

Cell culture supernate samples require a 2-fold dilution. A suggested 2-fold dilution 75 µL of sample + 75 µL of Calibrator Diluent RD5-20 (1X).

Serum and plasma samples require a 6-fold dilution. A suggested 6-fold dilution 20 µL of sample + 100 µL of Calibrator Diluent RD5-20 (1X).

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REAGENT PREPARATION

Bring all reagents to room temperature before use.

Mouse MFG-E8 Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the Control undiluted.

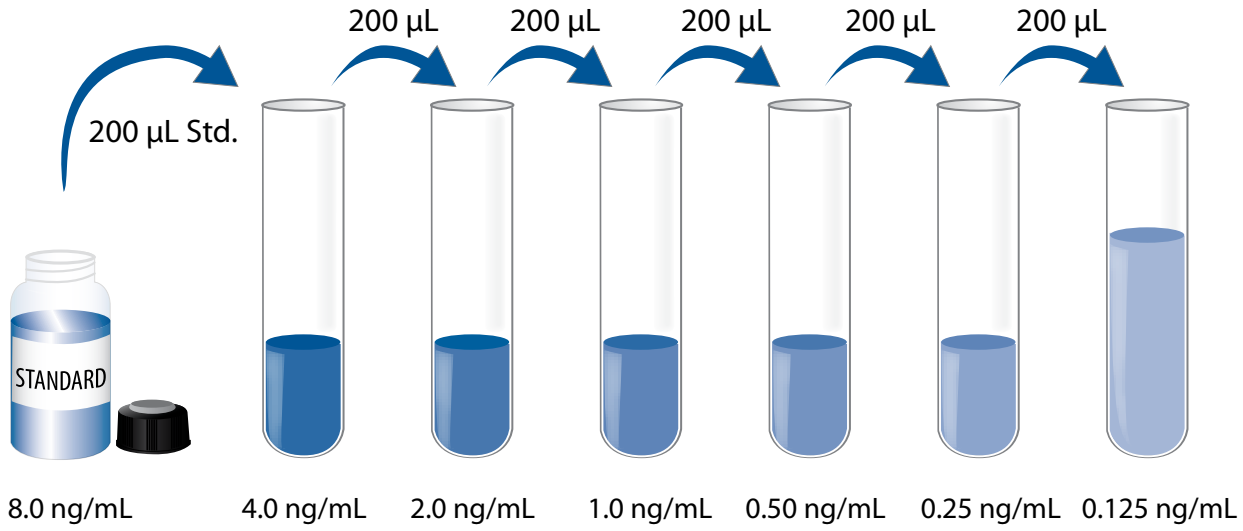
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-20 (1X) - Add 20 mL of Calibrator Diluent RD5-20 Concentrate to 60 mL of deionized or distilled water to prepare 80 mL of Calibrator Diluent RD5-20 (1X).

Mouse MFG-E8 Standard - Reconstitute the Mouse MFG-E8 Standard with 5.0 mL of Calibrator Diluent RD5-20 (1X). This reconstitution produces a stock solution of 8 ng/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 200 µL of Calibrator Diluent RD5-20 (1X) into each of six tubes. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Mouse MFG-E8 Standard serves as the high standard (8 ng/mL). Calibrator Diluent RD5-20 (1X) serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

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

1. Prepare all reagents, working standards, Control, 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 RD1-55 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 ± 50 rpm.
5. Aspirate each well and wash, repeating the process four times for a total of five 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 towels.
6. Add 100 µL of Mouse MFG-E8 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 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 100 µL of Stop Solution to each well. 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 require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

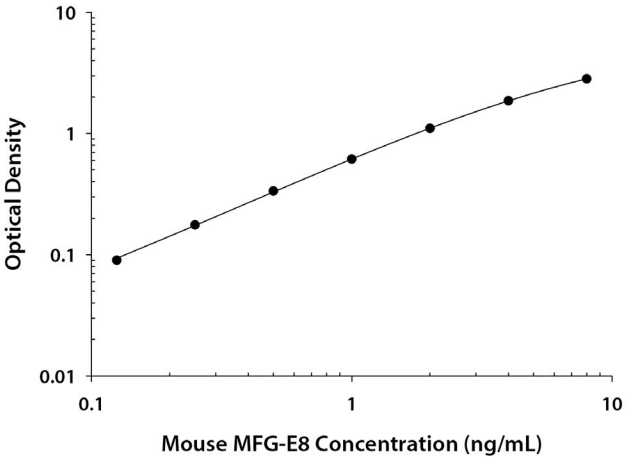
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 mouse MFG-E8 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.

Since 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.



(ng/mL)	O.D.	Average	Corrected
0	0.072 0.074	0.073	—
0.125	0.162 0.164	0.163	0.090
0.25	0.248 0.249	0.249	0.176
0.50	0.404 0.411	0.408	0.335
1.0	0.685 0.689	0.687	0.614
2.0	1.159 1.191	1.175	1.102
4.0	1.929 1.943	1.936	1.863
8.0	2.881 2.913	2.897	2.824

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 components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	0.506	0.936	3.89	0.509	0.910	3.78
Standard deviation	0.020	0.042	0.116	0.047	0.071	0.322
CV (%)	4.0	4.5	3.0	9.2	7.8	8.5

RECOVERY

The recovery of MFG-E8 spiked into various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates* (n=4)	99	90-104%
Serum* (n=4)	101	97-104%
Heparin plasma* (n=4)	94	90-98%

*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 mouse MFG-E8 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)	Heparin plasma* (n=4)
1:2	Average % of Expected	101	100	101
	Range (%)	97-106	99-102	99-104
1:4	Average % of Expected	104	98	105
	Range (%)	98-114	95-101	102-108
1:8	Average % of Expected	105	98	103
	Range (%)	96-119	94-101	96-107
1:16	Average % of Expected	103	96	103
	Range (%)	93-116	91-101	100-106

*Samples were diluted prior to assay.

SENSITIVITY

Forty assays were evaluated and the minimum detectable dose (MDD) of mouse MFG-E8 ranged from 0.003-0.020 ng/mL. The mean MDD was 0.009 ng/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 NS0-expressed recombinant mouse MFG-E8 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of MFG-E8 in this assay.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=20)	6.92	4.96-9.06	1.3
Heparin plasma (n=20)	5.61	0.516-9.54	2.7

Cell Culture Supernates - Organs from mice were removed, rinsed in 1X PBS, and kept on ice in 1X PBS. Organs were then homogenized and seeded into media containing RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 1 µg/mL lipopolysaccharide for 3 days. Aliquots of the cell culture supernates were removed and assayed for levels of natural MFG-E8.

Tissue Type	Unstimulated (ng/mL)	Stimulated (ng/mL)
Heart	0.406	0.396
Kidney	1.83	1.54
Liver	ND	ND
Lung	12.9	11.2
Spleen	3.82	3.16

ND=Non-detectable

SPECIFICITY

This assay recognizes natural and recombinant mouse MFG-E8.

The factors listed below were prepared at 500 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 500 ng/mL in a mid-range recombinant mouse MFG-E8 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:
EGF
EG-VEGF/PK1
Neuropilin-1
$VEGF_{120}$
VEGF R2/Flk-1

Recombinant rat:
Neuropilin-2

Recombinant human:
Integrin αVβ3
Neuropilin-2

Recombinant human MFG-E8 cross-reacts approximately 0.02% in this assay.

Rat serum samples were evaluated in this kit. No detectable levels were observed.

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