Quantikine® ELISA

Human MFG-E8 Immunoassay

Catalog Number DFGE80

For the quantitative determination of human Milk Fat Globulin Protein E8 (MFG-E8) concentrations in cell culture supernates, serum, plasma, saliva, urine, and human milk.

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 human MFG-E8 contains one N-terminal EGF-like domain and two C-terminal F5/8-type discoidin-like domains (2). It shares 63% and 61% amino acid (aa) sequence identity with comparable regions of mouse and rat MFG-E8, respectively. Shorter isoforms of human MFG-E8 may have N-terminal deletions (beginning near the end of the first discoidin-like domain), internal deletions (lacking either the EGF-like domain or the central region of the second discoidin-like domain), or C-terminal deletions (truncated within the second discoidin-like domain) (3-5). A 50 aa internal proteolytic fragment of MFG-E8 (known as Medin) is a major component of aortic medial amyloid deposits (6).

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 (7, 8). It is also found in multiple other cell types including endothelial cells and smooth muscle cells of the vasculature (9), immature dendritic cells (10, 11), at the acrosomal cap of testicular and epididymal sperm (12), and in epithelial cells of the endometrium (13).

An RGD motif within the EGF-like domain mediates MFG-E8 binding to the Integrins αVβ3 and αVβ5 (9, 14, 15). 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 (9, 16). The second discoidin-like domain mediates the binding of MFG-E8 to phosphatidylserine (PS) and other phospholipids (15, 17, 18). MFG-E8 functions as a bridge between PS on apoptotic cells and Integrin αVβ3 on phagocytes, leading to the clearance of apoptotic thymocytes (10, 18). 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 (19, 20). It reduces inflammation and disease progression in colitis by preventing Osteopontin from binding and activating Integrin αVβ3 (21). MFG-E8 binding to PS on the surface of injured intestinal epithelial cells promotes their migration and the regeneration of epithelial integrity (22). 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 (23, 24). MFG-E8 also promotes the removal of excess Collagen in fibrotic lungs (25), limits gut injury after ischemia-reperfusion (26), and blocks rotavirus infection (27). Its tissue-protective role also impairs anti-tumor immunity and chemotherapy-induced apoptosis (28).

The Quantikine® Human MFG-E8 Immunoassay is a 4.5 hour solid phase ELISA designed to measure human MFG-E8 in cell culture supernates, serum, plasma, saliva, urine, and human milk. It contains NS0-expressed recombinant human MFG-E8 and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate recombinant human MFG-E8. Results obtained using natural human MFG-E8 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 MFG-E8.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human MFG-E8 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any MFG-E8 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human 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 and color develops in proportion to the amount of MFG-E8 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 the appropriate 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.
- 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.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human MFG-E8 Microplate	894418	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human MFG-E8.	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 MFG-E8 Standard	894420	Recombinant human MFG-E8 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
Human MFG-E8 Conjugate	894419	21 mL of a monoclonal antibody specific for human MFG-E8 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-57	895207	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-20	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	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.
- 100 mL and 500 mL graduated cylinders.
- 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.
- Human MFG-E8 Controls (optional; R&D Systems®, Catalog # QC120).

PRECAUTIONS

MFG-E8 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 SDS 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.*
Hemolyzed samples are not recommended 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.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, and assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and centrifuge twice more for a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Cell culture supernate and urine samples require a 2-fold dilution. A suggested 2-fold dilution is 150 µL of sample + 150 µL of Calibrator Diluent RD5-20 (diluted 1:6)*.

Serum and plasma samples require a 6-fold dilution. A suggested 6-fold dilution is 50 µL of sample + 250 µL of Calibrator Diluent RD5-20 (diluted 1:4)*.

Saliva samples require a 2-fold dilution. A suggested 2-fold dilution is 150 µL of sample + 150 µL of Calibrator Diluent RD5-20 (diluted 1:4)*.

Human milk samples require a 10,000-fold dilution. A suggested 10,000-fold dilution can be achieved by adding 10 µL of sample to 990 µL of Calibrator Diluent RD5-20 (diluted 1:6)*. Complete the 10,000-fold dilution by adding 10 µL of the diluted sample to 990 µL of Calibrator Diluent RD5-20 (diluted 1:6)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High concentrations of MFG-E8 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 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

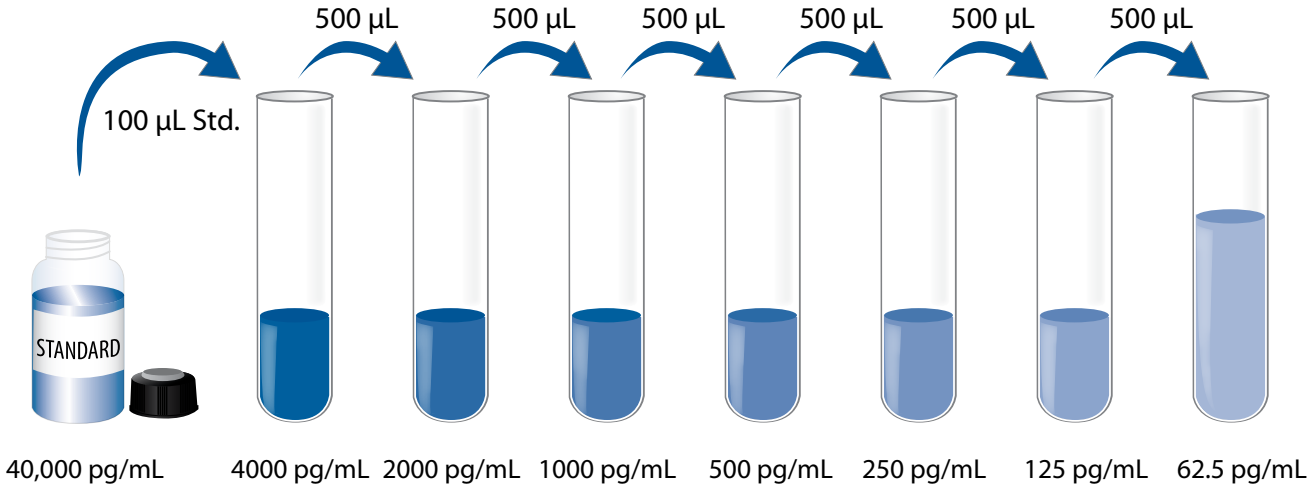
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-20 (diluted 1:4) - **For serum/plasma/saliva samples.** Add 4.0 mL of Calibrator Diluent RD5-20 to 12 mL of deionized or distilled water to yield 16 mL of Calibrator Diluent RD5-20 (diluted 1:4).

Calibrator Diluent RD5-20 (diluted 1:6) - **For cell culture supernate/urine/human milk samples.** Add 14 mL of Calibrator Diluent RD5-20 to 70 mL of deionized or distilled water to yield 84 mL of Calibrator Diluent RD5-20 (diluted 1:6).

Human MFG-E8 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human MFG-E8 Standard with deionized or distilled water. This reconstitution produces a stock solution of 40,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 900 µL of Calibrator Diluent RD5-20 diluted (1:4) (*for serum/plasma/saliva samples*) or Calibrator Diluent RD5-20 (diluted 1:6) (*for cell culture supernate/urine/human milk samples*) into the 4000 pg/mL tube. 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 4000 pg/mL standard 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, controls, and samples be assayed in duplicate.

Note: *High concentrations of MFG-E8 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 100 µL of Assay Diluent RD1-57 to each well.
4. Add 100 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. A plate layout is provided to record 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 Human 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 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 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.).

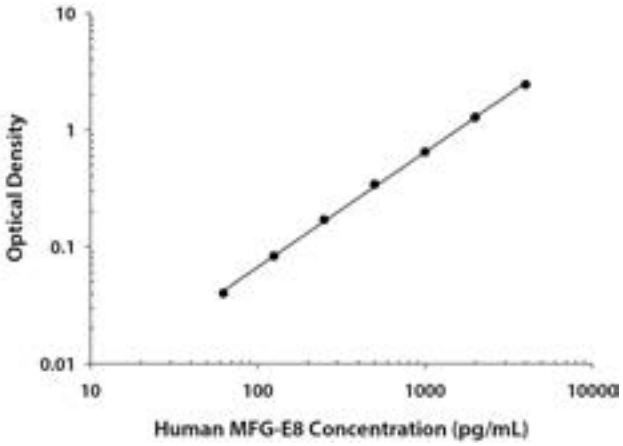
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human MFG-E8 concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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

TYPICAL DATA

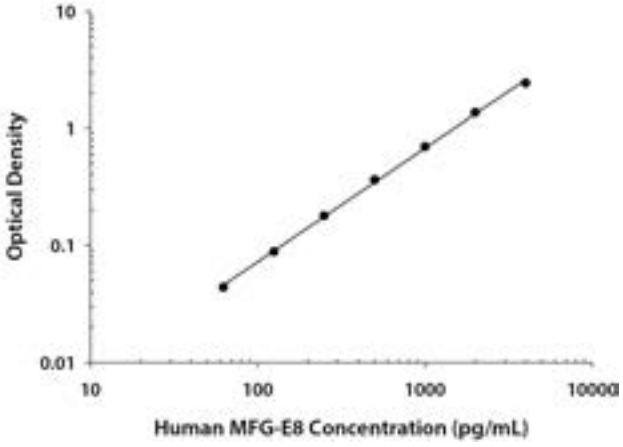
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

SERUM/PLASMA/SALIVA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.016 0.021	0.019	—
62.5	0.057 0.061	0.059	0.040
125	0.100 0.103	0.102	0.083
250	0.187 0.191	0.189	0.170
500	0.359 0.362	0.361	0.342
1000	0.657 0.675	0.666	0.647
2000	1.290 1.304	1.297	1.278
4000	2.418 2.479	2.449	2.430

CELL CULTURE SUPERNATE/URINE/HUMAN MILK ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.019 0.021	0.020	—
62.5	0.063 0.064	0.064	0.044
125	0.105 0.111	0.108	0.088
250	0.198 0.199	0.199	0.179
500	0.376 0.387	0.382	0.362
1000	0.709 0.725	0.717	0.697
2000	1.349 1.406	1.378	1.358
4000	2.406 2.474	2.440	2.420

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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.

CELL CULTURE SUPERNATE/URINE/HUMAN MILK ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	680	1181	2103	692	1262	2206
Standard deviation	30.5	38.9	84.7	57.6	88.0	125
CV (%)	4.5	3.3	4.0	8.3	7.0	5.7

SERUM/PLASMA/SALIVA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	645	1143	2168	749	1326	2339
Standard deviation	39.5	46.6	81.1	57.4	78.0	117
CV (%)	6.1	4.1	3.7	7.7	5.9	5.0

RECOVERY

The recovery of human MFG-E8 spiked to levels throughout the range of the assay in various matrices was evaluated. Samples were diluted prior to assay.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	105	100-110%
Serum (n=4)	101	92-110%
EDTA plasma (n=4)	96	89-102%
Heparin plasma (n=4)	96	89-102%

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of human MFG-E8 were serially diluted with the appropriate calibrator diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to assay.

		Cell culture supernates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Saliva (n=4)	Human milk (n=4)
1:2	Average % of Expected	100	105	109	108	94	106
	Range (%)	94-111	98-110	106-112	101-114	89-99	104-108
1:4	Average % of Expected	106	106	111	106	93	106
	Range (%)	99-113	100-113	106-112	103-108	88-100	103-108
1:8	Average % of Expected	105	101	111	103	96	105
	Range (%)	98-110	97-110	105-116	98-107	89-109	104-106
1:16	Average % of Expected	102	101	100	94	96	105
	Range (%)	94-109	90-112	89-112	88-103	84-114	100-110

SENSITIVITY

Sixty-one assays were evaluated and the minimum detectable dose (MDD) of human MFG-E8 ranged from 1.19-13.4 pg/mL. The mean MDD was 4.04 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 NS0-expressed recombinant human MFG-E8 produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human MFG-E8 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=40)	3835	2041-7406	1310
EDTA plasma (n=40)	4630	2349-8549	1466
Heparin plasma (n=40)	4114	1979-8379	1450
Saliva (n=12)	2372	848-6126	1759

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Urine (n=12)	359	92	ND-922

ND=Non-detectable

Sample Type	Mean (µg/mL)	Range (µg/mL)	Standard Deviation (µg/mL)
Human milk (n=15)	14.1	5.41-51.1	11.2

Cell Culture Supernates:

MCF 10A human breast epithelial cells were cultured in 50% F-12/50% DMEM supplemented with 5% equine serum, 100 ng/mL cholera enterotoxin, 10 µg/mL insulin, 0.5 µg/mL hydrocortisol, and 20 ng/mL recombinant human EGF. An aliquot of the cell culture supernate was removed, assayed for human MFG-E8, and measured 7221 pg/mL.

A549 human lung carcinoma cells were cultured in F-12 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. An aliquot of the cell culture supernate was removed, assayed for human MFG-E8, and measured 18,220 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human MFG-E8.

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 MFG-E8 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

EGF
pro-EGF
EGFR
EG-VEGF
FGF-3
FGF-12
FGF-16
FGF-17
FGF-19
FGF-20
FGF-22
FGF acidic
Integrin α6
Integrin αMβ2
Integrin αVβ3
Integrin αVβ5
MSP
Neuropilin-1
Neuropilin-2
PlGF
$VEGF_{121}$
$VEGF_{162}$
$VEGF_{165}$
$VEGF\text{-}B_{167}$
VEGF-C
VEGF-D
VEGF/PlGF
VEGF R1
VEGF R2
VEGF R3

Recombinant mouse:

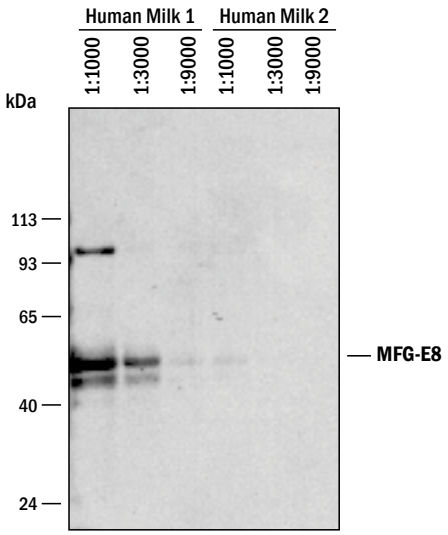
EGF
pro-EGF
EG-VEGF
MFG-E8
Neuropilin-1
PlGF-2
$VEGF_{120}$
$VEGF_{164}$
$VEGF\text{-}B_{167}$
VEGF-D
VEGF R1
VEGF R2
VEGF R3

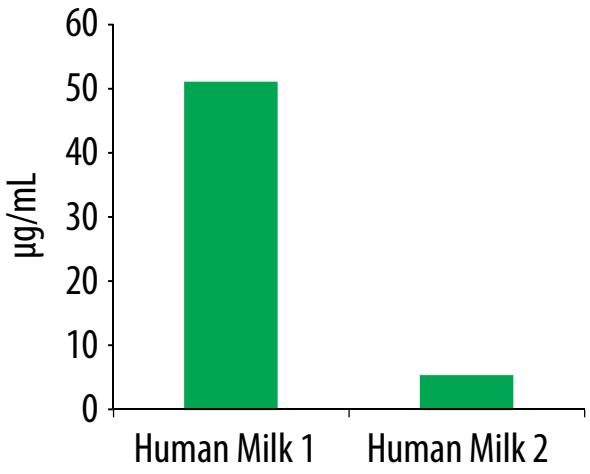
Recombinant rat:

EGF
Neuropilin-1
Neuropilin-2
$VEGF_{164}$

Other recombinants:

canine KGF/FGF-7
zebrafish $VEGF_{165}$





Two different human milk samples were analyzed by Western Blot and Quantikine® ELISA. The samples were diluted 1:1000, 1:3000, and 1:9000, resolved under reducing SDS-PAGE conditions, transferred to a PVDF membrane, and immunoblotted with a sheep anti-human MFG-E8 antibody. The Western Blot shows a direct correlation with the ELISA value for these samples.

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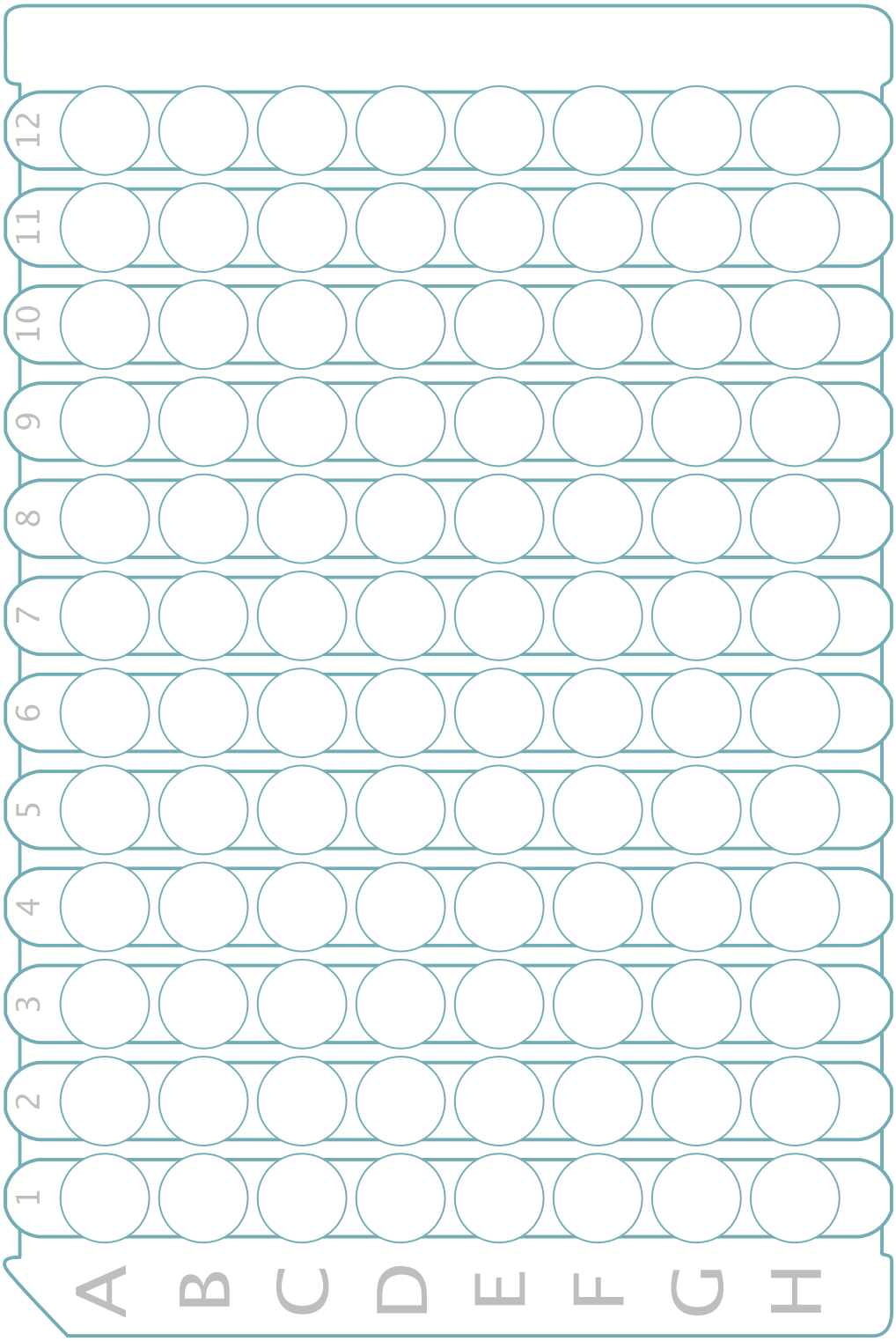
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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