Quantikine® ELISA

GDF-8/Myostatin

Quantikine® ELISA

GDF-8/Myostatin Immunoassay

Catalog Number DGDF80

For the quantitative determination of Growth Differentiation Factor 8 (GDF-8) concentrations in cell culture supernates, tissue homogenates, 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

Growth Differentiation Factor 8 (GDF-8), also known as Myostatin, is a secreted TGF-β superfamily protein that is expressed in developing and adult skeletal muscle. It controls myoblast proliferation and is a potent negative regulator of skeletal muscle mass (1). GDF-8 is synthesized as a 376 amino acid (aa) preproprotein that consists of a 24 aa signal peptide, a 243 aa propeptide, and a 109 aa mature protein (2). The secreted proprotein is cleaved by BMP-1 family proteases to separate the 35-40 kDa propeptide from the 12 kDa bioactive mature protein (3-6). This cleavage results in a latent complex containing a disulfide-linked dimer of the mature protein and two noncovalently-associated propeptides (2, 5). Within the propeptide, mouse GDF-8 shares 96% and 99% aa sequence identity with human and rat GDF-8, respectively. The amino acid sequence of mature GDF-8 is 100% conserved between human, mouse, and rat.

In the latent complex, the GDF-8 propeptide functions as an inhibitor of mature GDF-8. GDF-8 activity can also be inhibited through its association with Follistatin, FLRG, Decorin, or GASP-1 (5, 7-11). GDF-8 additionally binds with high affinity to GASP-2/WFIKKN (12). The uncleaved GDF-8 proprotein binds Latent TGF-β bp3 which can sequester it in the extracellular matrix and prevent the proteolytic cleavage of the propeptide (13). Genetic deletion of GDF-8 as well as *in vivo* administration or overexpression of the propeptide increase skeletal muscle fiber hypertrophy but not proliferation (2, 14, 15). Active GDF-8 binds to the type II Activin receptor Activin RIIB which then associates with the type I receptors Activin RIB/ALK-4 or TGF-β RI/ALK-5 to induce signaling (16).

GDF-8 is upregulated in cardiomyocytes surrounding infarcted areas after mycardial infarction (17). It is released by mechanically-stressed cardiomyocytes and induces the skeletal muscle wasting which is common in heart failure (18, 19). Circulating levels of GDF-8 are elevated following pressure overload-induced cardiac hypertrophy (18). Genetic GDF-8 inactivation or blockade leads to multiple changes in energy balance and metabolism including increased fatty acid oxidation and brown fat formation (20), reduced white fat accumulation and circulating cholesterol and triglyceride levels (21-23), and increased peripheral insulin sensitivity and resistance to diet-induced obesity (21-25).

The Quantikine® GDF-8/Myostatin Immunoassay is a 4.5 hour solid phase ELISA designed to measure GDF-8 levels in cell culture supernates, tissue homogenates, serum, and plasma. It contains NS0-expressed recombinant GDF-8 and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant GDF-8 accurately. Results obtained using natural GDF-8 showed dose-response 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 GDF-8.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mature GDF-8 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells, and any GDF-8 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for mature GDF-8 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 GDF-8 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 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. 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
GDF-8 Microplate	894408	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for GDF-8.	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.*
GDF-8 Conjugate	894409	21 mL of a monoclonal antibody specific for GDF-8 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
GDF-8 Standard	894410	Recombinant GDF-8 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-17	895433	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. *Use diluted 1:4 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of a 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 activation of samples.
- GDF-8 Controls (optional; R&D Systems®, Catalog # QC98).

ADDITIONAL REAGENTS REQUIRED

For sample activation:

- Hydrochloric acid (A.C.S. Grade, 12 N)
- Sodium hydroxide (A.C.S. Grade, 10 N)
- HEPES, free acid (Reagent Grade, M.W. 238.3)

PRECAUTIONS

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.

Note: *Samples containing fetal bovine serum are not suitable for use in this assay. Other animal serum used in the preparation of cell culture media may contain high levels of GDF-8. For best results, do not use animal serum for growth of cell cultures when assaying for GDF-8 production. If animal serum is used as a supplement in the media, precautions should be taken to prepare the appropriate control and run the control in the immunoassay to determine the baseline concentration of GDF-8.*

Tissue Homogenates - Prior to assay, tissues must be homogenized according to the directions in the Sample Values section.

Human 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.

Mouse 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.

Rat/Porcine/Canine/Equine Serum - Allow blood samples to clot for 2 hours at room temperature before centrifugation for 30 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 and lipemic samples are not suitable for use in this assay.

ACTIVATION REAGENT PREPARATION

To remove the pro-peptide from GDF-8, prepare the following solutions for acid activation and neutralization. The solutions may be stored in polypropylene bottles at room temperature for up to one month.

Caution: *Wear protective clothing and safety glasses during preparation or use of these reagents. Refer to the appropriate MSDS prior to use.*

1 N HCl (100 mL) - To 91.67 mL of deionized water, slowly add 8.33 mL of 12 N HCl. Mix well.

1.2 N NaOH/0.5 M HEPES (100 mL) - To 75 mL of deionized water, slowly add 12 mL of 10 N NaOH. Mix well. Add 11.9 g of HEPES. Mix well. Bring final volume to 100 mL with deionized water.

For each new lot of acidification and neutralization reagents, measure the pH of several representative samples after neutralization to ensure that it is within pH 7.2-7.6. Adjust the volume and corresponding dilution factor of the neutralization reagent as needed.

SAMPLE ACTIVATION PROCEDURE

To activate GDF-8 to immunoreactive GDF-8 detectable by the Quantikine® GDF-8 immunoassay, follow the activation procedure outlined below. Assay samples after neutralization (pH 7.2-7.6). **Use polypropylene test tubes.**

Note: ***Do not activate the kit standards or controls.*** *The kit standards and controls contain mature recombinant GDF-8. Use the chart below for volumes of 1N HCl, 1.2 N NaOH/0.5 M HEPES, and Calibrator Diluent RD5-26 (diluted 1:4)* used for specific sample types.*

1. Add 1N HCl to sample. Mix well. Incubate for 10 minutes at room temperature.
2. Add 1.2 N NaOH/0.5 M HEPES. Mix well.
3. Add Calibrator Diluent RD5-26 (diluted 1:4)*. Mix well and assay within 2 hours.

Note: *The concentration read off of the standard curve must be multiplied by the appropriate dilution factor.*

SAMPLE PREPARATION

Due to high endogenous values, samples require additional dilution after sample activation.

Sample Type	Sample (µL)	1 N HCl (µL)	1.2 N NaOH/ 0.5 M HEPES (µL)	Calibrator Diluent RD5-26 (diluted 1:4) (µL)	Final Dilution Factor
Serum-free cell culture supernates	100	50	50	200	1:4
Human serum & plasma	100	50	50	200	1:4
Mouse serum & plasma	20	10	10	760	1:40
Rat/Canine serum & plasma	20	10	10	360	1:20
Porcine/Equine serum & plasma	20	10	10	160	1:10
Tissue homogenates	20	10	10	160	1:10

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

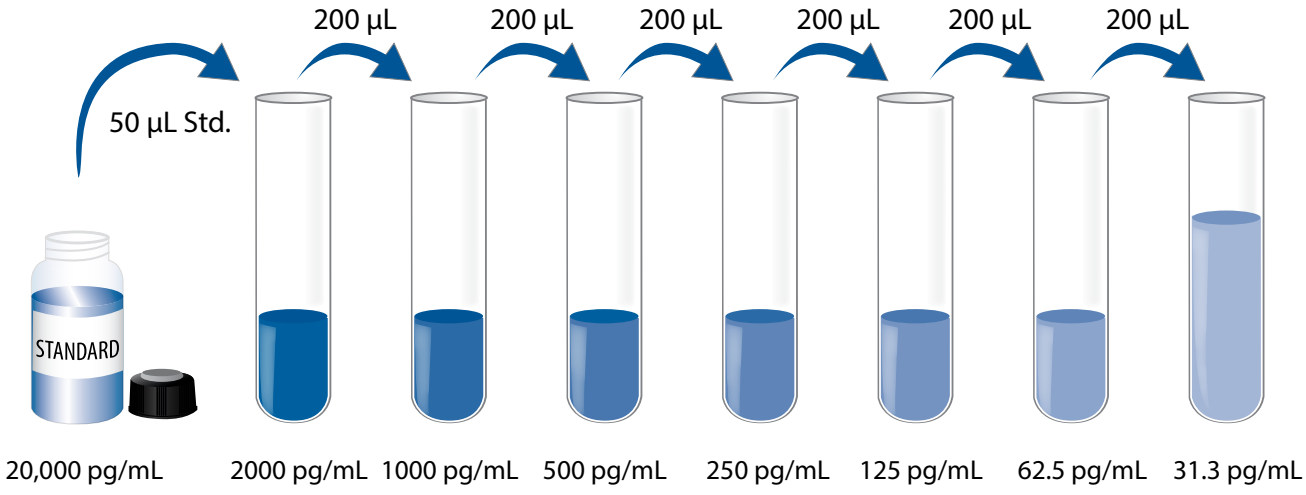
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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.

Calibrator Diluent RD5-26 (diluted 1:4) - Add 20 mL of Calibrator Diluent RD5-26 Concentrate to 60 mL of deionized or distilled water to prepare 80 mL of Calibrator Diluent RD5-26 (diluted 1:4).

GDF-8 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the GDF-8 Standard with deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5-26 (diluted 1:4) into the 2000 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-26 (diluted 1:4) 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, 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 RD1-17 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. 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 towels.
6. Add 200 µL of GDF-8 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. 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.

*Samples require activation and dilution. See Sample Activation and Sample Preparation sections.

CALCULATION OF RESULTS

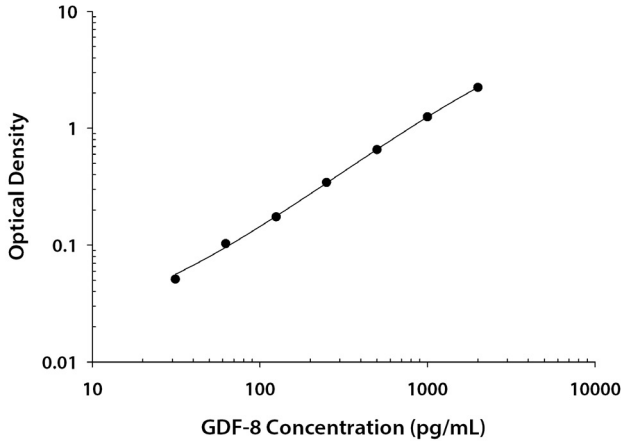
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 GDF-8 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 activated and 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	0.016 0.017	0.017	—
31.3	0.066 0.069	0.068	0.051
62.5	0.121 0.119	0.120	0.103
125	0.189 0.193	0.191	0.174
250	0.352 0.369	0.361	0.344
500	0.660 0.681	0.671	0.654
1000	1.241 1.294	1.268	1.251
2000	2.218 2.277	2.248	2.231

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 (pg/mL)	171	583	1147	146	491	1058
Standard deviation	9.20	14.8	20.9	8.8	17.7	32.3
CV (%)	5.4	2.5	1.8	6.0	3.6	3.1

RECOVERY

The recovery of GDF-8 spiked to levels throughout the range of the assay in various matrices was evaluated.

Human Samples	Average % Recovery	Range
Cell culture samples (n=4)	110	100-118%
Tissue homogenates (n=4)	100	90-120%
Serum & Plasma (n=12)	91	80-117%

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of GDF-8 were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to assay.

Human		Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	104	98	102
	Range (%)	96-115	88-102	97-106
1:4	Average % of Expected	107	102	109
	Range (%)	100-115	97-106	98-118
1:8	Average % of Expected	110	105	111
	Range (%)	104-118	103-109	107-120
1:16	Average % of Expected	110	107	113
	Range (%)	105-115	103-109	108-119

Note: *Mouse, rat, canine, porcine, and equine samples were evaluated and no significant difference in recovery or linearity was observed from the data above.*

SENSITIVITY

Thirty assays were evaluated and the minimum detectable dose (MDD) of GDF-8 ranged from 0.922-5.32 pg/mL. The mean MDD was 2.25 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 mature GDF-8 (aa 268-376 of accession # O08689) produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of GDF-8 in this assay.

	Serum (pg/mL)		
Species	Mean	Range	Standard Deviation
Human (n=35)	4206	1264-8588	1906
Mouse (n=10)	36,064	21,680-47,360	8539
Rat (n=10)	9778	4240-15,880	3199
Porcine (n=10)	9218	3170-17,110	3846
Canine (n=10)	10,688	2720-17,520	4897
Equine (n=4)	4407	968-9860	4160

	EDTA plasma (pg/mL)		
Species	Mean	Range	Standard Deviation
Human (n=35)	3531	1220-7300	1556
Mouse (n=5)	22,304	19,200-27,160	3223
Rat (n=5)	9180	8420-10,320	757
Canine (n=5)	11,992	7560-16,380	3560

	Heparin plasma (pg/mL)		
Species	Mean	Range	Standard Deviation
Human (n=35)	3454	1200-7640	1542
Mouse (n=5)	32,752	23,760-47,800	9227
Rat (n=5)	13,700	10,960-17,120	2548
Canine (n=5)	14,320	7720-19,220	5038

SAMPLE VALUES *CONTINUED*

Cell Culture Supernates - 3T3-L1 mouse embryonic fibroblast adipose-like cells were cultured in DMEM supplemented with 10% fetal bovine serum and grown for 3 days until confluent. Cells were transferred to serum-free DMEM and cultured for an additional 24 hours. Aliquots of the culture supernates were removed and assayed for levels of GDF-8. No detectable levels were observed.

Tissue Homogenates - Tissue from mice were rinsed with PBS, chopped into 1-2 mm pieces, and homogenized with a tissue homogenizer in PBS. Sample was frozen and thawed twice at -80 °C. Debris was then removed by centrifugation. Aliquots of the lysate were removed and assayed for levels of GDF-8.

Tissue Type	(pg/mL)
Brain	ND
Skeletal Muscle	1720

ND=Non-detectable

SPECIFICITY

This assay recognizes natural and recombinant mature GDF-8.

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 GDF-8 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Activin RIIA
Activin RIIB
Decorin
GASP-2
GDF-11
GDF-15

Recombinant mouse:

GDF-1 (mature)
GDF-1 (propeptide)
GDF-3 (mature)
GDF-3 (propeptide)
GDF-5
GDF-6
GDF-7
GDF-9 (prepro)
GDF-9 (propeptide)
GDF-9 (mature)
GDF-15
FLRG

Recombinant human Follistatin and recombinant human GASP-1 interfere at levels > 10 ng/mL in this assay.

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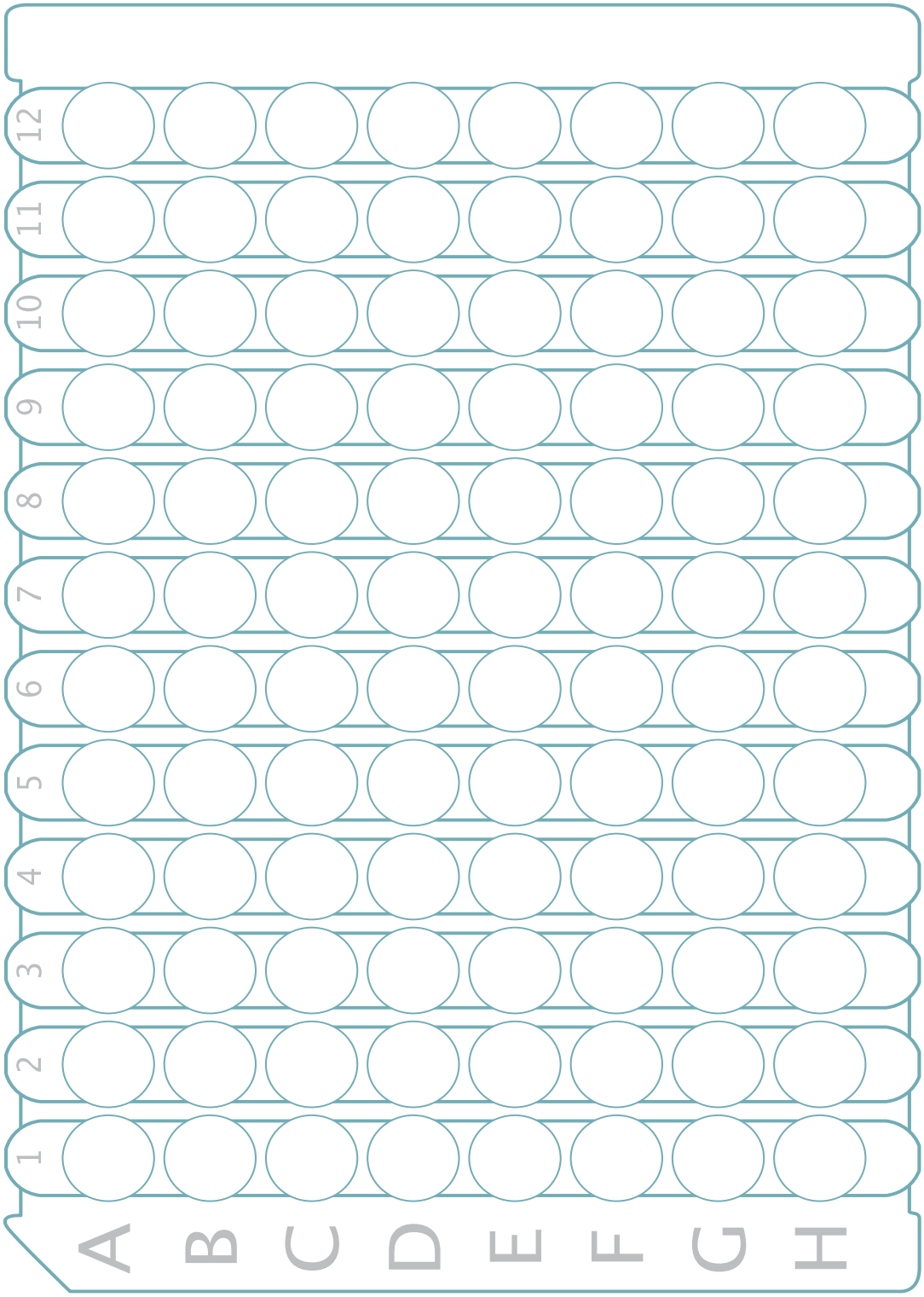
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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