Quantikine® ELISA

Human FLRG Immunoassay

Catalog Number DFLRG0

For the quantitative determination of human Follistatin-Related Gene (FLRG) concentrations in cell culture supernates, serum, plasma, 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

Follistatin-related Gene (FLRG), also known as Follistatin-like 3 (FSTL3) and Follistatin-related protein (FSRP), is a 30-35 kDa secreted glycoprotein that exerts a broad range of activities by neutralizing Activin A and Myostatin function (1). Mature human FLRG consists of an Activin and Myostatin binding N-terminal domain, two Follistatin-like domains, and two Kazal-like domains (2-5). BMP-2, -6, and -7 do not compete for Activin A binding, and FLRG binds only weakly to Activin B (6, 7). Unlike Follistatin, FLRG does not contain a heparin-binding domain and does not interact with heparan sulfate proteoglycans (8, 9). A short form of FLRG, which lacks the signal sequence and is found in the nucleus, retains Activin-blocking capability (10, 11). Nuclear FLRG binds to the transcription factor AF10, induces AF10 homo-oligomerization, and enhances AF10-dependent gene transcription (12). Mature human FLRG shares 84% amino acid sequence identity with mouse and rat FLRG.

FLRG expression is induced by Activin A, providing negative feedback regulation of Activin A function (13, 14). FLRG is expressed in the uterus (15, 16), ovary (14, 17), testis (18), placenta (19), trophoblast (20), and adrenal gland (17). It is upregulated in cardiac myocytes following myocardial injury and blocks the prosurvival and anti-hypertrophic effects of Activin A on these cells (21, 22). FLRG is also expressed by astroglial cells in areas of neuronal trauma (23). Its binding to ADAM12 as well as to Activin A inhibits osteoclastic differentiation from macrophages (24). The Activin-neutralizing function enables FLRG to regulate energy balance and metabolism, as shown in FLRG knockout mice which exhibit increased pancreatic islet number and size, improved glucose tolerance and insulin sensitivity, and a shift from visceral to subcutaneous fat (25, 26). Obese ob/ob mice show FLRG downregulation in visceral fat and upregulation in subcutaneous fat relative to wild type mice (27). Circulating FLRG levels are decreased in women with gestational diabetes (28, 29). FLRG is upregulated in breast cancer and promotes tumor cell proliferation (30, 31).

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

PRINCIPLE OF THE ASSAY

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

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 SDS on our website prior to use.

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 FLRG Microplate	894134	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human FLRG.	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 FLRG Conjugate	894135	21 mL of a monoclonal antibody specific for human FLRG conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human FLRG Standard	894136	Recombinant human FLRG 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 RD5-24	895325	21 mL of a buffered protein base with preservatives. *Use diluted 1:3 for cell culture supernate/urine samples. Use undiluted for serum/plasma/human milk samples.*	
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 FLRG Controls (optional; R&D Systems®, Catalog # QC91).

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 heparin or EDTA 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.*

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

Serum and plasma samples require at least a 5-fold dilution. A suggested 5-fold dilution can be achieved by adding 100 µL of sample to 400 µL of Calibrator Diluent RD5-24.

Urine samples require at least a 2-fold dilution. A suggested 2-fold dilution can be achieved by adding 150 µL of sample to 150 µL of Calibrator Diluent RD5-24 (diluted 1:3)*.

Human milk samples require at least a 2-fold dilution. A suggested 2-fold dilution can be achieved by adding 150 µL of sample to 150 µL of Calibrator Diluent RD5-24.

*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 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

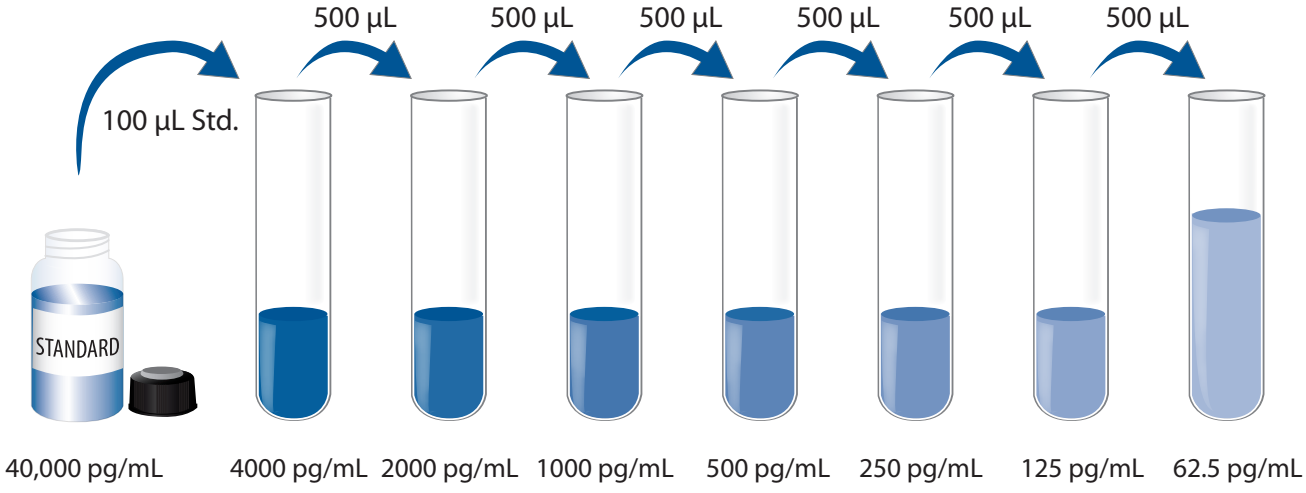
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-24 (diluted 1:3) - **For use with cell culture supernate/urine samples.** Add 5.0 mL of Calibrator Diluent RD5-24 to 10 mL of deionized or distilled water to prepare Calibrator Diluent RD5-24 (diluted 1:3).

Note: *Use undiluted Calibrator Diluent RD5-24 with serum, plasma, and human milk samples.*

Human FLRG Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human FLRG Standard with deionized or distilled water. This reconstitution produces a stock solution of 40,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5-24 (diluted 1:3) (*for cell culture supernate/urine samples*) or Calibrator Diluent RD5-24 (*for serum, plasma, and 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.

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 RD1W 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 ± 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 Human FLRG 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 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.).

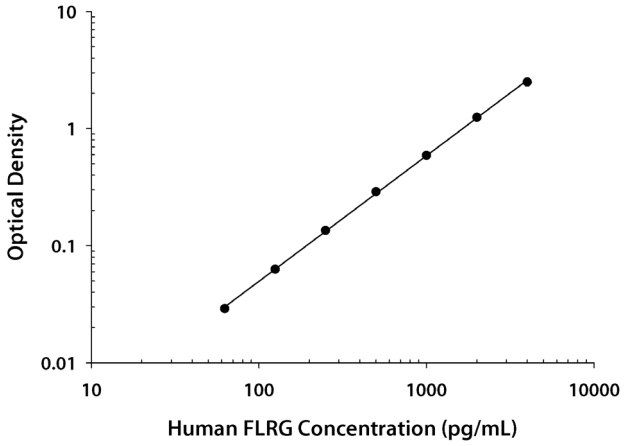
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points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

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

TYPICAL DATA

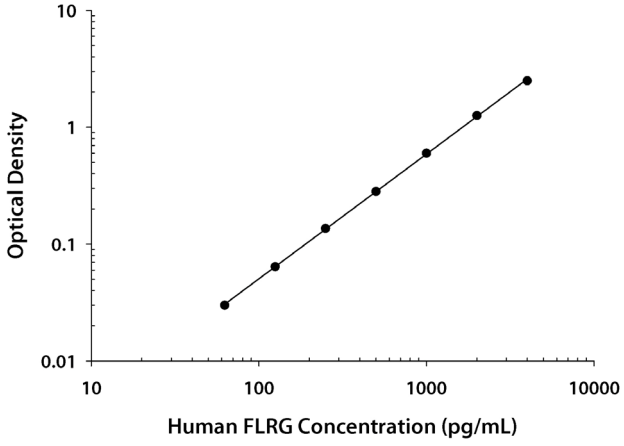
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/URINE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.011 0.012	0.012	—
62.5	0.040 0.041	0.041	0.029
125	0.074 0.075	0.075	0.063
250	0.145 0.148	0.147	0.135
500	0.299 0.302	0.301	0.289
1000	0.598 0.605	0.602	0.590
2000	1.254 1.262	1.258	1.246
4000	2.498 2.519	2.509	2.497

SERUM/PLASMA/HUMAN MILK ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.010 0.010	0.010	—
62.5	0.039 0.040	0.040	0.030
125	0.074 0.074	0.074	0.064
250	0.144 0.148	0.146	0.136
500	0.281 0.303	0.292	0.282
1000	0.603 0.614	0.609	0.599
2000	1.257 1.278	1.268	1.258
4000	2.480 2.534	2.507	2.497

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.

CELL CULTURE SUPERNATE/URINE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	468	1113	2252	494	1174	2331
Standard deviation	10.4	23.0	55.2	32.9	69.0	143
CV (%)	2.2	2.1	2.5	6.7	5.9	6.1

SERUM/PLASMA/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)	448	1094	2306	486	1148	2282
Standard deviation	15.4	18.5	49.5	29.5	53.0	122
CV (%)	3.4	1.7	2.1	6.1	4.6	5.3

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	107	98-111%
Serum* (n=4)	100	91-112%
EDTA Plasma* (n=4)	96	85-105%
Heparin plasma* (n=4)	102	89-114%

*Samples were diluted prior to assay.

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of human FLRG were serially diluted with the appropriate 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)	Urine* (n=4)	Human milk* (n=4)
1:2	Average % of Expected	104	98	99	98	104	102
	Range (%)	100-107	96-100	94-102	92-101	97-109	98-107
1:4	Average % of Expected	104	100	100	101	105	104
	Range (%)	100-108	98-102	100-101	100-102	97-112	100-112
1:8	Average % of Expected	106	101	101	102	104	102
	Range (%)	99-111	98-105	97-104	99-105	94-111	98-107
1:16	Average % of Expected	104	99	99	100	100	——
	Range (%)	95-111	96-101	97-106	97-102	90-106	——

*Samples were diluted prior to assay.

SENSITIVITY

Fifty assays were evaluated and the minimum detectable dose (MDD) of human FLRG ranged from 2.01-8.55 pg/mL. The mean MDD was 3.68 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 FLRG produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma/Urine/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of human FLRG 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=38)	6481	3142-8554	1192
EDTA plasma (n=38)	6338	3329-8808	1192
Heparin plasma (n=38)	6257	3214-8953	1172
Urine (n=11)	1144	171-2382	773
Human milk (n=16)	605	142-2028	556

Cell Culture Supernates:
JAR human choriocarcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37 °C until confluent. An aliquot of the cell culture supernate was removed, assayed for human FLRG, and measured 8690 pg/mL.

IMR-90 human lung fibroblast cells were cultured in MEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine. An aliquot of the cell culture supernate was removed, assayed for human FLRG, and measured 4054 pg/mL.

SW13 human adrenal cortex adenocarcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, and 2 mM L-glutamine, and incubated in 5% CO_2 at 37 °C until confluent. An aliquot of the cell culture supernate was removed, assayed for human FLRG, and measured 362 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human FLRG.

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

Recombinant human:

Activin C
Activin RIA/ALK-2
Activin RIIA
Activin RIIB
ADAM12
ALK-1
BAMBI/NMA
BMP-1/PCP
BMP-2
BMP-3
BMP-3b/GDF-10
BMP-4
BMP-5
BMP-6
BMP-7
BMP-8b
BMP-10
BMP-15
BMPR-IA/ALK-3
BMPR-IB/ALK-6
BMPR-II
Cripto
DAN
Endoglin/CD105
Fibronectin
Follistatin 288
Follistatin 300
Follistatin 315
Lefty A
Inhibin A
Inhibin B
LAP (TGF-β1)
MIS/AMH
Osteoactivin/GPNMB
TGF-α
TGF-β1
TGF-β1.2
TGF-β2
TGF-β3
TGF-β RII
TGF-β RIII

Recombinant mouse:

Activin C
Activin RIB
Activin RIIB
ALK-1
BAMBI/NMA
BMP-3b/GDF-10
BMPR-IA/ALK-3
BMPR-IB/ALK-6
Cripto
DAN
Endoglin/CD105
FLRG
Lefty-1
MIS/AMH
Noggin
Osteoactivin/GPNMB

Recombinant rat:

Agrin
ALK-7
MIS/AMH

Other recombinants:

zebrafish BMP-2
amphibian TGF-β5
porcine TGF-β2

Natural proteins:

human TGF-β1
porcine TGF-β1

The following recombinant factors did not show any significant cross-reactivity with human FLRG in this immunoassay, but demonstrated a low level of interference. These factors were added at various concentrations to a mid level human FLRG control. The chart below lists the percentage the human FLRG control decreased out of the +/-3 SD range in the presence of the indicated concentration of interfering factor:

Concentration	Human Activin A	Human/Mouse/Rat GDF-8/Myostatin	Human Activin AB	Human Activin B
12.5 ng/mL	0.40%	——	——	——
25 ng/mL	1.50%	0.90%	1.50%	3.10%
50 ng/mL	6.90%	7.00%	5.30%	13.30%

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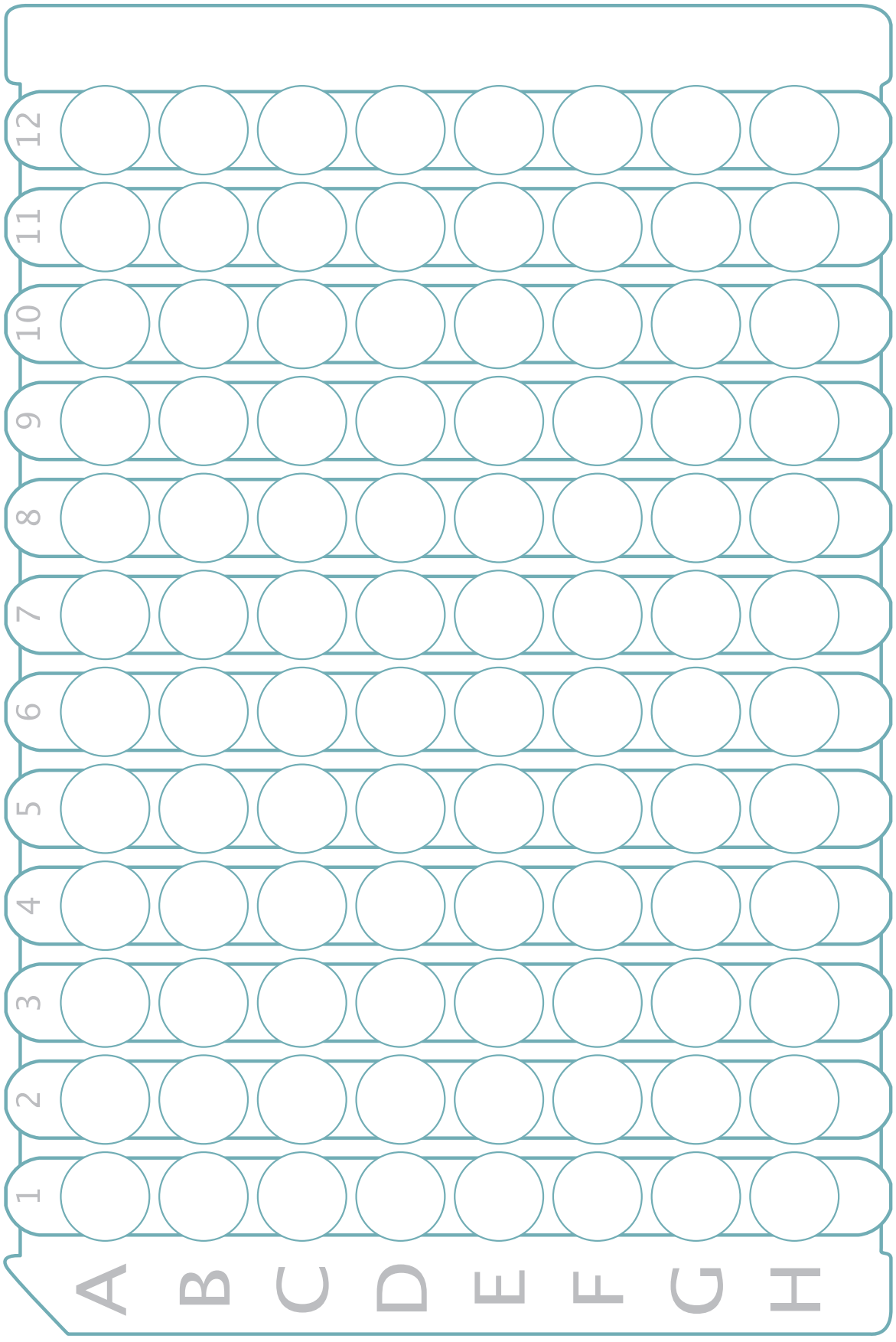
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES