Quantikine® ELISA

Human IGFBP-3 Immunoassay

Catalog Number DGB300
SGB300
PDGB300

For the quantitative determination of human Insulin-like Growth Factor Binding Protein 3 (IGFBP-3) 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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614 McKinley Place NE, Minneapolis, MN 55413, USA
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INTRODUCTION

Insulin-like Growth Factor Binding Proteins (IGFBPs) play a pivotal role in the regulation of IGF activity and availability in the circulation and extracellular environment. All IGFBP family members are cysteine-rich proteins with high binding affinity for IGF-I and IGF-II. IGFBPs can inhibit IGF actions, enhance IGF actions, or function independently as cell regulatory factors (for reviews, see references 1-4). IGFBPs act as transporters of IGF, prolonging the life of IGF within the body. IGFBP-3, the major IGF binding protein in plasma, exists in a ternary complex with IGF-I or IGF-II and an acid-labile subunit (ALS).

The full-length cDNA for IGFBP-3 encodes a precursor protein of 291 amino acids (aa) with a putative 27 aa signal peptide (5). Cleavage of the signal peptide generates the 264 aa mature protein. IGFBP-3 contains three potential N-linked and two potential O-linked glycosylation sites (6). Post-translational modifications such as glycosylation, phosphorylation and/or proteolysis may significantly influence the regulation of IGFBP-3 physiology (for reviews, see references 3 and 7). Studies have also suggested that the three-dimensional structure of the IGFBP-3 protein itself plays an important role in IGF binding (8-11). Substitutions for hydrophobic amino acids within the N-terminal domain of IGFBP-3 markedly reduce IGF-I binding and attenuate inhibition of IGF-I-stimulated cell migration and DNA synthesis (10). Residues 228-232 of IGFBP-3 are essential for cell association and are required for normal ALS binding affinity (11).

IGFBP-3 is expressed in multiple tissues. Circulating IGFBP-3 originates mainly from hepatic non-parenchymal cells. Expression levels are higher during extrauterine life and peak during puberty. Circulating levels of IGFBPs are regulated by both hormonal and metabolic factors (4). For example, prolonged periods of severe malnutrition can result in depressed levels of IGFBP-3 (12). Cytokines have also been shown to regulate IGFBP-3 expression. EGF and TGF-β1, growth factors known to be active in skin, can significantly reduce IGFBP-3 expression by keratinocytes (13). TGF-β1 can also regulate IGFBP-3 expression by glomerular endothelial cells, fetal chondrocytes, intestinal muscle cells, and fibroblasts (14-17).

Proteolysis of IGFBP-3 lowers its affinity for IGF, thus increasing the overall ratio of free IGF to total bound IGF and releasing fragments of IGFBP-3. IGFBP-3 is extensively degraded in serum during pregnancy. ADAM12, a disintegrin metalloprotease, has IGFBP-3 protease activity and may contribute to the IGFBP-3 degradation observed in serum during pregnancy (18). IGFBP-3 is also susceptible to proteolysis by the tissue plasminogen activator (tPA)-plasminogen-plasmin system (19-21). The neutrophil proteases, cathepsin G and elastase, both demonstrate proteolytic cleavage of IGFBP-3 (and other IGFBPs) thus suggesting that they may act to regulate IGFs and IGFBPs during inflammation and wound healing (22). Prostate specific antigen (PSA) is also an IGFBP-3 protease. PSA can decrease IGFBP-3 affinity for IGF, potentiate IGF action in the presence of inhibitory IGFBP-3 and, as a result, potentially contribute to normal and malignant prostate growth (23, 24).

IGFBP-3 also has IGF-independent effects on cell growth. Although IGFBP-3 is a potent inhibitor of IGF activity, it also has IGF-independent anti-proliferative effects by facilitating apoptosis (25, 26).

The tumor suppressor p53, a critical mediator of apoptosis in response to cellular stress, can induce expression of the IGFBP-3 gene (27). This induction presents a connection between p53 and the IGF axis. IGFBP-3 may serve to protect against potentially carcinogenic effects of growth hormone and IGF-I (27, 28).

Serum levels of IGFBP-3 can be used to screen for growth hormone deficiency (29-31). Serum levels of IGFBP-3 are decreased in AIDS, uncontrolled diabetes mellitus, trauma, and severe burns (32).

The Quantikine® Human IGFBP-3 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human IGFBP-3 in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant human IGFBP-3 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human IGFBP-3 showed linear curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that the Quantikine® Human IGFBP-3 kit can be used to determine relative mass values for natural human IGFBP-3.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human IGFBP-3 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IGFBP-3 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human IGFBP-3 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 IGFBP-3 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.
- 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 #	CATALOG # DGB300	CATALOG # SGB300	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human IGFBP-3 Microplate	890887	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human IGFBP-3.	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 IGFBP-3 Conjugate	890888	1 vial	6 vials	21 mL of a polyclonal antibody specific for human IGFBP-3 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human IGFBP-3 Standard	890889	1 vial	6 vials	Recombinant human IGFBP-3 in a buffer with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-62	895330	1 vial	6 vials	11 mL of a buffered protein base with preservatives. *May appear cloudy. Mix well before and during use.*	
Calibrator Diluent RD5P	895151	1 vial	6 vials	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:5 in this assay.*	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	6 vials	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

DGB300 contains sufficient materials to run an ELISA on one 96 well plate.
SGB300 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PDGB300). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Refer to the literature accompanying your order for specific vial counts.

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.
- 2-8 °C refrigerator.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human IGFBP-3 Controls (optional; R&D Systems®, Catalog # QC23).

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.

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.*

SAMPLE PREPARATION

Use polypropylene tubes.

Serum and plasma samples require a 100-fold dilution. A recommended 100-fold dilution is 10 µL of sample + 990 µL of Calibrator Diluent RD5P (diluted 1:5)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents (except conjugate) to room temperature before use.

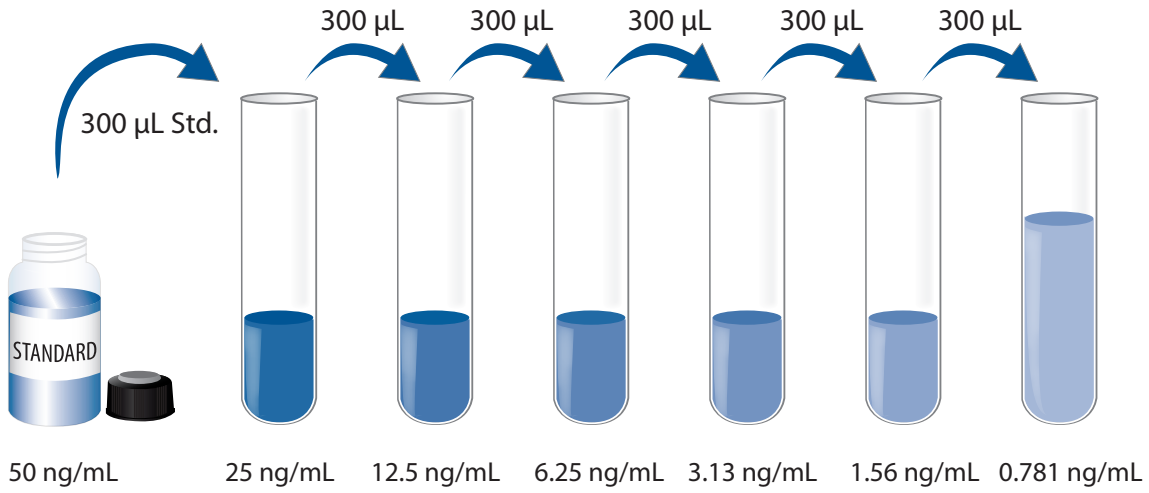
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

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 RD5P (diluted 1:5) - Add 20 mL of Calibrator Diluent RD5P to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5P (diluted 1:5).

Human IGFBP-3 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human IGFBP-3 Standard with Calibrator Diluent RD5P (diluted 1:5). This reconstitution produces a stock solution of 50 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 300 µL of Calibrator Diluent RD5P (diluted 1:5) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Human IGFBP-3 Standard (50 ng/mL) serves as the high standard. Calibrator Diluent RD5P (diluted 1:5) serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Conjugate should remain at 2-8 °C until use. Bring all other 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 100 µL of Assay Diluent RD1-62 to each well. *Assay Diluent RD1-62 may appear cloudy. Warm to room temperature and mix gently until solution appears uniform.*
4. Add 100 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours **at 2-8 °C.** 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 auto washer. 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 **cold** Human IGFBP-3 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours **at 2-8 °C.**
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. 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

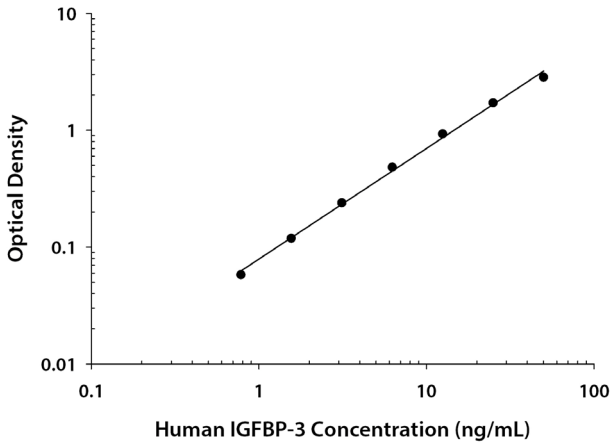
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a 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 IGFBP-3 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.



(ng/mL)	O.D.	Average	Corrected
0	0.015 0.018	0.016	—
0.781	0.070 0.063	0.066	0.050
1.56	0.114 0.111	0.112	0.096
3.13	0.220 0.207	0.214	0.198
6.25	0.408 0.395	0.402	0.386
12.5	0.741 0.779	0.760	0.744
25	1.436 1.413	1.424	1.408
50	2.569 2.401	2.485	2.469

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 forty 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	40	40	40
Mean (ng/mL)	4.13	12.7	31.1	4.06	11.8	28.5
Standard deviation	0.20	0.64	0.71	0.22	0.76	2.27
CV (%)	4.8	5.0	2.3	5.4	6.4	8.0

RECOVERY

The recovery of human IGFBP-3 spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	94	89-99%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human IGFBP-3 were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture samples (n=4)	Serum* (n=5)	EDTA plasma* (n=5)	Heparin plasma* (n=5)
1:2	Average % of Expected	102	104	100	101
	Range (%)	97-112	102-107	93-110	92-107
1:4	Average % of Expected	99	105	106	106
	Range (%)	93-108	102-106	95-113	97-113
1:8	Average % of Expected	98	103	107	106
	Range (%)	91-109	100-105	96-112	97-109
1:16	Average % of Expected	96	103	106	107
	Range (%)	88-103	100-107	90-111	100-114

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

SENSITIVITY

Forty-four assays were evaluated and the minimum detectable dose (MDD) of human IGFBP-3 ranged from 0.02-0.14 ng/mL. The mean MDD was 0.05 ng/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 IGFBP-3 produced at R&D Systems®.

The NIBSC non-WHO reference material 93/560 recombinant human IGFBP-3 was evaluated in this assay. The dose response curve parallels the Quantikine® standard curve. To convert sample values obtained with the Quantikine® Human IGFBP-3 kit to approximate NIBSC 93/560 values, use the equation below.

NIBSC (93/560) approximate value (ng/mL) = 1.0131 x Quantikine® Human IGFBP-3 value (ng/mL)

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human IGFBP-3 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=70)	2375	835-3778	541
EDTA Plasma (n=35)	2175	1430-3311	421
Heparin Plasma (n=35)	2301	1553-3089	372

Cell Culture Supernates:
PC-3 human prostate cancer cells were grown to confluency in RPMI supplemented with 5% fetal bovine serum. Cells were cultured unstimulated or stimulated with 10 ng/mL of recombinant human TNF-α. Aliquots of the cell culture supernates were removed and assayed for human IGFBP-3.

Condition	Concentration (ng/mL)
Unstimulated	11.2
Stimulated	37.4

A431 human epithelial carcinoma cells were grown to confluency in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. An aliquot of the cell culture supernate was removed, diluted 20-fold with Calibrator Diluent RD5P (diluted 1:5), assayed for human IGFBP-3, and measured 448 ng/mL.

A549 human lung carcinoma cells were grown to confluency in K-F12 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. An aliquot of the cell culture supernate was removed, assayed for human IGFBP-3, and measured 12.2 ng/mL.

HepG2 human hepatocellular carcinoma cells were grown to confluency in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. An aliquot of the cell culture supernate was removed, diluted 2-fold with Calibrator Diluent RD5P (diluted 1:5), assayed for human IGFBP-3, and measured 71.4 ng/mL.

IMR-90 human lung fibroblasts were grown to confluency in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. An aliquot of the cell culture supernate was removed, diluted 4-fold with Calibrator Diluent RD5P (diluted 1:5), assayed for human IGFBP-3, and measured 110 ng/mL.

MRC-5 human embryonic lung fibroblasts were grown to confluency in RPMI supplemented with 10% fetal bovine serum and 2 mM L-glutamine. An aliquot of the cell culture supernate was removed, assayed for human IGFBP-3, and measured 23.9 ng/mL.

4MBr-5 rhesus monkey epithelial cells were grown to confluency in F12 medium supplemented with 10% fetal bovine serum, 50 ng/mL of recombinant human EGF, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. An aliquot of the cell culture supernate was removed, diluted 10-fold with Calibrator Diluent RD5P (diluted 1:5), assayed for human IGFBP-3, and measured 224 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human IGFBP-3.

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 IGFBP-3 standard were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

β-ECGF
EGF
FGF acidic
FGF basic
FGF-4
FGF-5
FGF-6
FGF-9
FGF-10
FGF-18
Flt-3 Ligand
G-CSF
GM-CSF
HB-EGF
HGF
NRG1-α
IGF-I
IGF-II
IGF-I R
IGFBP-1
IGFBP-2
IGFBP-4
IGFBP-5
IGFBP-6
KGF
M-CSF
MSP
MSP β
β-NGF
PDGF-AA
PDGF-AB
PDGF-BB
PD-ECGF
PlGF
$VEGF_{121}$
$VEGF_{165}$
VEGF/PlGF
VEGF-D
VEGF R3/Flt-4

Recombinant mouse:

FGF-8b
FGF-8c
Flt-3 Ligand
G-CSF
GM-CSF
IGF-I
IGF-II
M-CSF
PlGF-2
$VEGF_{120}$
$VEGF_{164}$

Recombinant rat:

GM-CSF
β-NGF
PDGF-BB

Recombinant porcine:

GM-CSF

Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF

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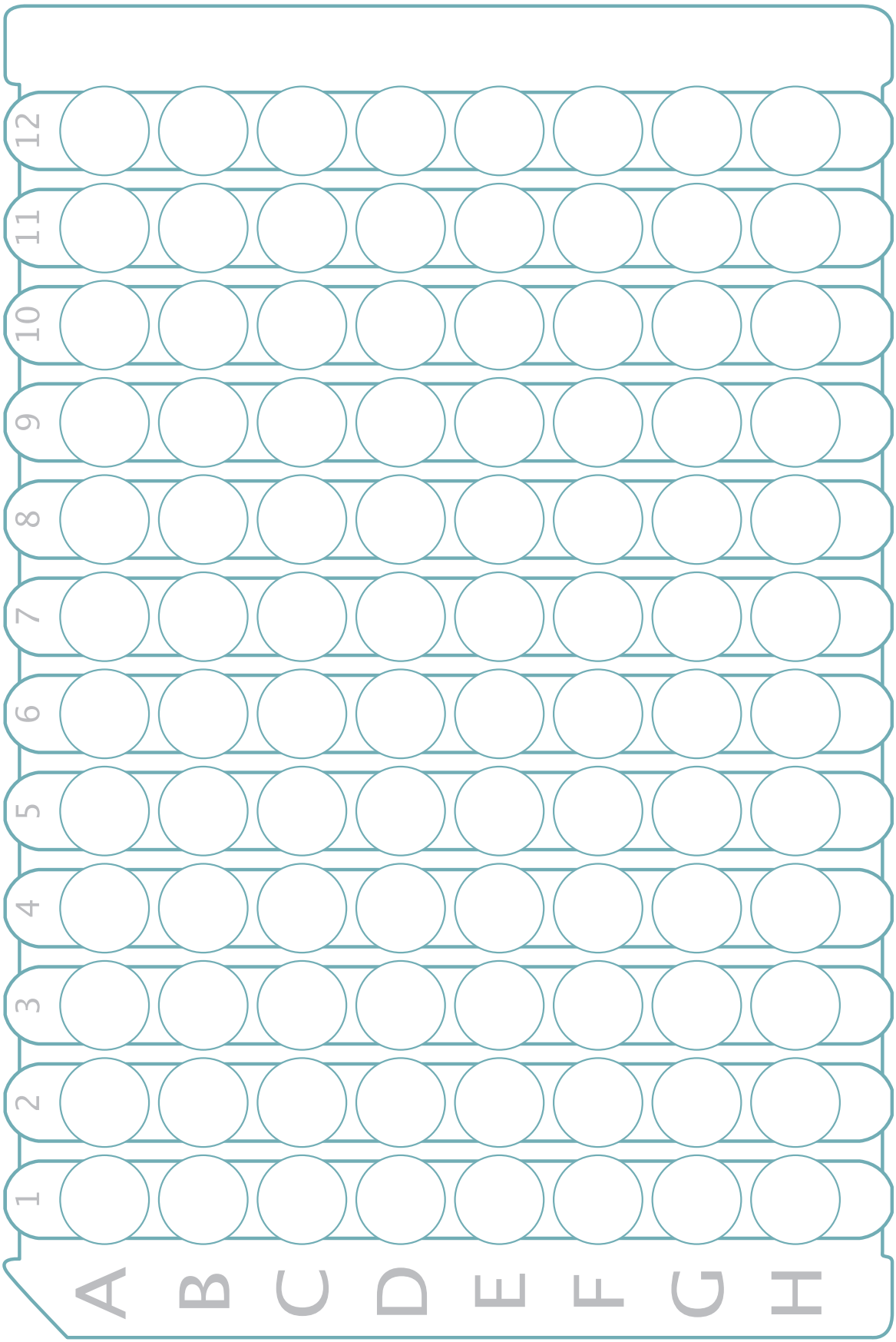
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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