Quantikine® ELISA

Human IGF-II Immunoassay

Catalog Number DG200

For the quantitative determination of human Insulin-like Growth Factor 2 (IGF-II) concentrations in cell culture supernates, serum, plasma, and urine.

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

Insulin-like Growth Factor-2 (IGF-II), also known as Somatomedin-A, is a glycosylated 8 kDa Insulin family peptide hormone. It is part of a complex system of growth and metabolic regulating proteins that is particularly important during development in the nervous system, adrenal cortex, and skeletal system (1, 2). Human IGF-II is synthesized as a preproprotein that contains a 24 amino acid (aa) signal sequence, a 67 aa mature region, and an 89 aa C-terminal prosegment, commonly referred to as the E domain (3, 4). Sequential proteolytic processing generates the 18 kDa ProIGF-II followed by mature IGF-II which consists of an N-terminal B domain followed by a C domain, an A domain, and a D domain (5, 6). The B-C-A sequence is reminescent of Proinsulin, which is processed further to remove the C domain. Mature human IGF-II shares 91% and 94% aa identity with mouse and rat IGF-II, respectively. Additional IGF-II related products include a splice isoform with a 56 aa N-terminal extension, "Big" IGF-II which retains 21 aa of the E domain, and Preptin (a 34 aa fragment of the E domain) which is secreted by pancreatic β-cells and facilitates both Insulin secretion and osteoblast proliferation (7-9).

IGF-II is primarily synthesized by the liver and circulates in both fetus and adult (1, 2). In the blood, mature IGF-II associates with IGFBP-1, 2, 4 or 6 and can form a ternary complex with either IGFBP-3 or IGFBP-5 plus ALS (Acid-Labile Subunit). It can additionally bind to soluble IGF-II R (which may carry 20% of total circulating IGF-II) (10, 11). ProIGF-II also circulates and can form a ternary complex with IGFBP-5 and ALS (6, 11). Once dissociated from its carrier protein, mature IGF-II has mitogenic, antiapoptotic, and Insulin like activities on a wide variety of cell types. It binds and activates IGF-I R, IGF-II R, Insulin R (both A and B isoforms), and IGF-I R:Insulin R-A hybrid receptors (6, 10, 12-15).

The Quantikine® Human IGF-II Immunoassay is a 3.5 hour solid-phase ELISA designed to measure human IGF-II in cell culture supernates, serum, plasma, and urine. It contains *E. coli*-expressed recombinant human IGF-II and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human IGF-II 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 IGF-II.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human IGF-II has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IGF-II present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human IGF-II 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 IGF-II 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, 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 #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human IGF-II Microplate	898500	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human IGF-II.	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 IGF-II Standard	898502	2 vials of recombinant human IGF-II in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new standard for each assay. Discard after use.
Human IGF-II Conjugate Concentrate	898501	300 µL of a concentrated polyclonal antibody specific for human IGF-II conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C after dilution.*
Conjugate Diluent 33	896071	21 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-42	895565	3 vials (21 mL/vial) of a buffered protein base with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Pretreatment G	898503	6 mL of 15 mg/mL glycine, pH 2.0.	
Pretreatment H	898504	6 mL of buffer 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.
- 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 IGF-II Controls (optional; R&D Systems®, Catalog # QC230).

PRECAUTIONS

IGF-II is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

Pretreatment H is toxic if swallowed, inhaled, or in contact with skin. Do not eat or drink when using this product. Do not breathe fumes. Use only in a well-ventilated area.

The Stop Solution and Pretreatment G provided with this kit are acid solutions.

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: *Animal serum used in the preparation of cell culture media may contain high levels of animal IGF-II that shares a high sequence homology. For best results, use serum-free media for growth of cell cultures during the last 24 hours when assaying for IGF-II production.*

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.*

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.

SAMPLE PREPARATION

Follow the sample pretreatment and dilution procedure outlined below in order to dissociate IGF binding proteins from IGF-II.

Cell Culture Supernates/Urine	Serum/Plasma
To 50 µL of cell culture supernate, add 50 µL of Pretreatment G.	To 10 µL serum/plasma, add 95 µL of Pretreatment G.
Mix well.	Mix well.
Incubate 10 minutes at room temperature.	Incubate 10 minutes at room temperature.
Neutralize the acidified sample by adding 50 µL of Pretreatment H.	Neutralize the acidified sample by adding 95 µL of Pretreatment H.
Mix well.	Mix well.
Prior to assay, cell culture supernate samples may require dilution. Urine samples require a 2-fold dilution. A suggested 2-fold dilution is 75 µL of pretreated sample + 75 µL of Calibrator Diluent RD5-42. Assay samples within 60 minutes of pretreatment.	Prior to assay, serum and plasma samples require a 100-fold dilution due to high endogenous levels. A suggested 100-fold dilution is 10 µL of pretreated sample + 990 µL of Calibrator Diluent RD5-42. Assay samples within 60 minutes of pretreatment.
The concentration read off the standard curve must be multiplied by the appropriate dilution factor for cell culture supernates, or by 6 for urine.	The concentration read off the standard curve must be multiplied by the dilution factor, 2000.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High concentrations of IGF-II are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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.

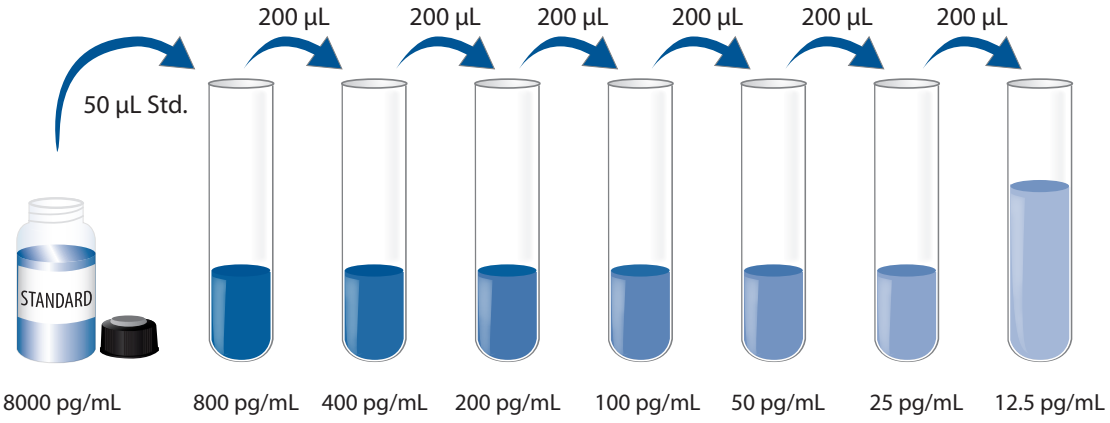
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Human IGF-II Conjugate (1X) - Add 0.215 mL of Human IGF-II Conjugate Concentrate directly to the Conjugate Diluent 33 vial. Mix well.

Human IGF-II Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human IGF-II Standard with deionized or distilled water. This reconstitution produces a stock solution of 8000 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.

Note: *Do not use rocker.*

Pipette 450 µL of Calibrator Diluent RD5-42 into the 800 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 800 pg/mL standard serves as the high standard. Calibrator Diluent RD5-42 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 samples, controls, and standards be assayed in duplicate.

Note: *High concentrations of IGF-II are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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 Calibrator Diluent RD5-42 to each well.
4. Add 50 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for **1 hour** at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided for a record of 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 IGF-II 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.

CALCULATION OF RESULTS

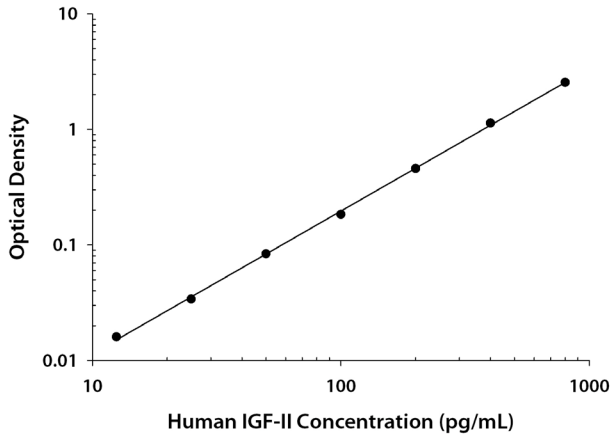
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 IGF-II concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

Because 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.



(pg/mL)	O.D.	Average	Corrected
0	0.010 0.014	0.012	—
12.5	0.028 0.028	0.028	0.016
25	0.046 0.046	0.046	0.034
50	0.093 0.099	0.096	0.084
100	0.195 0.197	0.196	0.184
200	0.466 0.475	0.471	0.459
400	1.121 1.166	1.144	1.132
800	2.560 2.575	2.568	2.556

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)	128	305	566	136	307	558
Standard deviation	4.05	7.20	15.6	9.89	28.5	28.2
CV (%)	3.2	2.4	2.8	7.3	9.3	5.1

RECOVERY

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

Sample Type	Average % Recovery	Range
Serum-free cell culture media* (n=4)	95	82-104%
Urine** (n=4)	94	79-104%

LINEARITY

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

		Serum-free cell culture media* (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)	Urine** (n=4)
1:2	Average % of Expected	105	98	102	105	106
	Range (%)	103-107	97-100	101-106	101-107	104-109
1:4	Average % of Expected	102	96	105	107	107
	Range (%)	97-105	94-97	101-109	106-110	105-110
1:8	Average % of Expected	102	94	110	110	108
	Range (%)	98-105	89-101	107-113	108-113	104-114
1:16	Average % of Expected	105	98	113	112	111
	Range (%)	100-109	95-101	111-115	109-114	105-119

*Samples were pretreated prior to assay.
**Samples were pretreated and diluted prior to assay.

SENSITIVITY

Twenty-nine assays were evaluated and the minimum detectable dose (MDD) of human IGF-II ranged from 0.718-5.41 pg/mL. The mean MDD was 1.88 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 *E. coli*-expressed recombinant human IGF-II manufactured at R&D Systems®.

The NIBSC/WHO IGF-II Reference Reagent 96/538 was evaluated in this kit. The dose response curve of the reference reagent 96/538 parallels the Quantikine® standard curve. To convert sample values obtained with the Quantikine® Human IGF-II kit to approximate NIBSC/WHO 96/538 Units, use the equation below.

NIBSC/WHO (96/538) approximate value (IU/mL) = 0.001 x Quantikine® IGF-II value (pg/mL)

Note: *Based on data generated in September 2016.*

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human IGF-II 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=30)	500	296-706	105
EDTA plasma (n=30)	411	268-655	101
Heparin plasma (n=30)	380	237-600	82.3

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Urine (n=10)	152	50	ND-236

ND=Non-detectable

Cell Culture Supernates:

HepG2 human hepatocellular carcinoma cells were cultured in MEM NEAA Earle's Salts supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 1 mM sodium pyruvate until they were 90% confluent. The cells were then washed with PBS and cultured for an additional 24 hours in serum-free MEM NEAA Earle's Salts supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 1 mM sodium pyruvate. An aliquot of the cell culture supernate was removed, assayed for human IGF-II, and measured 5895 pg/mL.

HT-29 human colon adenocarcinoma cells were cultured in McCoy's Medium 5A supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate, until they were 90% confluent. The cells were then washed with PBS and cultured for an additional 24 hours in serum-free McCoy's Medium 5A supplemented with 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 IGF-II, and measured 2940 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human IGF-II.

The factors listed below were prepared at 100 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 100 ng/mL in a mid-range recombinant human IGF-II control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Cyr61
Endocan
IGF-I R
IGF-II R
IGFBP-1
IGFBP-4
IGFBP-5
IGFBP-6
IGFBP-7
IGFBP-L1
IGF-I
IGFL-1
IGFL-3
IGFL-4
INSR
Insulin
Pro-Insulin
Transferrin
WISP-1

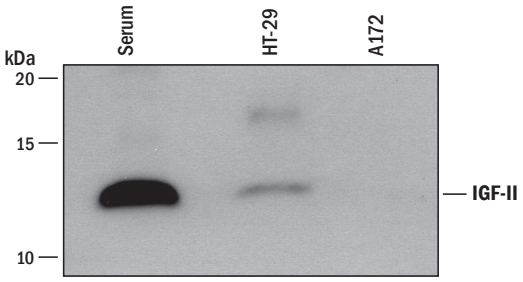
Natural proteins:

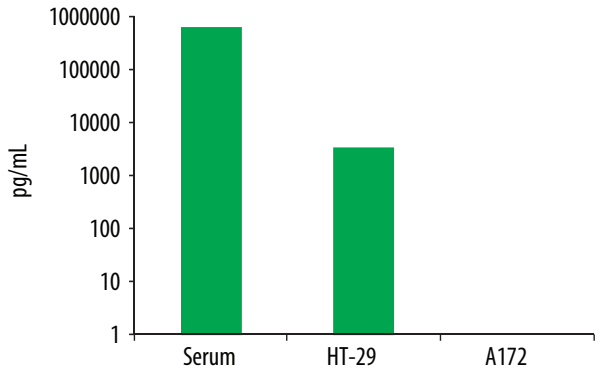
human Apo-Transferrin*
human Holo-Transferrin
human Plasminogen

*Prepared at 2.5 µg/mL.

Recombinant human (rh) IGFBP-2 and rhIGFBP-3 interfere at concentrations > 25 ng/mL.

Recombinant mouse IGF-II cross-reacts approximately 0.2% in this assay.





Human serum (diluted 1:50) and serum-free conditioned media from HT-29 and A172 cell lines were analyzed by Western Blot and ELISA. Samples were resolved under reducing SDS-PAGE conditions, transferred to a PVDF membrane, and immunoblotted with the detection antibody used in this kit. The Western Blot band intensity shows a direct correlation with ELISA sample values.

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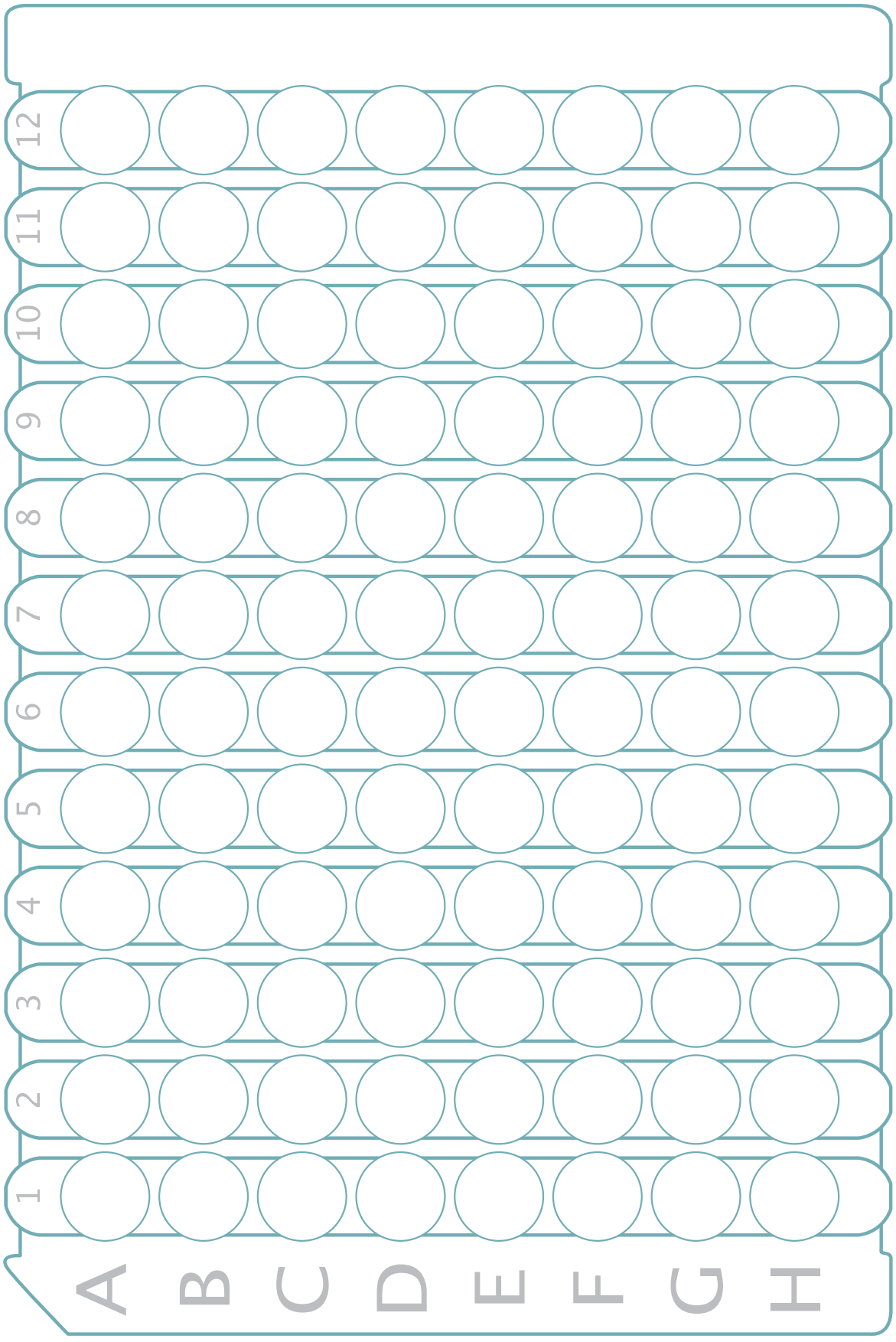
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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