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R&D SYSTEMS

Quantikine® ELISA

Human Haptoglobin Immunoassay

Catalog Number DHAPG0

For the quantitative determination of human Haptoglobin concentrations in serum-free cell culture supernates, serum, plasma, saliva, 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

Haptoglobin is a 45-48 kDa acute phase glycoprotein that is an inactive member of the peptidase S1 family of serine proteases (1, 2). It is predominantly secreted by the liver, but low levels are produced by multiple tissues outside the liver, including adipose, lung, spleen, kidney, and post-ischemic hippocampus (3-6). Its major function is as an antioxidant that protects tissues from hemoglobin-mediated oxidative damage (1, 7-9). The 388 amino acid (aa) human pro-Haptoglobin is proteolytically cleaved between Arg102-Ile103, while still within the endoplasmic reticulum, by serine proteases such as complement component C1rLP (9, 10). The resulting disulfide-linked α- and β-chains then dimerize through α-chain linkages to create a tetramer (2). A portion of circulating Haptoglobin is unprocessed pro-Haptoglobin. Human Haptoglobin shares 80% aa identity with mouse Haptoglobin.

Two alleles of Haptoglobin are found in humans, producing HP*1F (fast, 1-1), HP*1S (slow, 2-2) and HP*2 FS (heterozygous 1-2) phenotypes (1). The 2 allele produces a longer α-chain than the 1 allele (142 aa and 83 aa, respectively), and tetramers containing it are more likely to polymerize and less likely to infiltrate tissues (1). A rare deficiency of Haptoglobin (Hp 0-0) has also been described (1). Zonulin, an uncleaved form of allele 2, was independently discovered as a molecule that inhibits epithelial tight junctions, downregulating barrier function (9). Zonulin production in the small intestines is stimulated by triggers such as wheat glutens and certain enteric bacteria, and it is thought to contribute to excessive intestinal permeability in conditions such as celiac disease and Crohn's disease (9).

Haptoglobin shows extremely high-affinity association with hemoglobin (11). It detoxifies free hemoglobin dimers in the circulation by burying the oxidative portions and chaperoning hemoglobin to the scavenger receptor CD163 on the surface of monocytes and macrophages for clearance (2, 7-9, 11). Haptoglobin is also an acute phase plasma protein (1, 3). Its expression is induced by IL-6-type cytokines and synergistically enhanced by glucocorticoids, with peak increases a day after an inflammatory event (1, 3, 12). IL-6-induced Haptoglobin expression is enhanced by arterial shear stress and nitric oxide, and attenuated by insulin, HGF and EGF (3, 13). Haptoglobin has anti-inflammatory properties that are independent of hemoglobin binding (3). It inhibits prostaglandin synthesis, scavenges nitric oxide, dampens the inflammatory response to endotoxins, inhibits T cell proliferation, inhibits neutrophil respiratory bursts, binds neutrophil and monocyte integrin CD11b/CD18 ($\alpha_M\beta_2$ or MAC-1) and inhibits the binding of B cell CD22 to TNF-α-activated endothelial cells (1, 14-16). Purified Haptoglobin shows direct antimicrobial activity and is angiogenic (1, 4, 17). The 2 allele shows weaker hemoglobin binding, antioxidative capacity and inhibition of prostaglandin synthesis, but stronger affinity toward CD22 than the 1 allele (1, 18, 19). It is associated with more frequent onset of diabetic complications and cardiovascular disease (1, 18, 19). In mice, deletion of Haptoglobin facilitates the development of autoimmune inflammation (12).

The Quantikine® Human Haptoglobin Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Haptoglobin in serum-free cell culture supernates, serum, plasma, saliva, and urine.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Haptoglobin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Haptoglobin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Haptoglobin 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 Haptoglobin 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 #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Haptoglobin Microplate	894567	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Haptoglobin.	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 Haptoglobin Conjugate	894568	21 mL of a polyclonal antibody specific for human Haptoglobin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Haptoglobin Standard	894569	Human Haptoglobin in a buffered protein base with preservatives; lyophilized. **Note:** *Human sourced material. See Precautions section. Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-109	895966	2 vials (11 mL/vial) of a buffered protein base with preservatives.	
Calibrator Diluent RD5-67	896011	21 mL of a buffered protein base with preservatives. *Use diluted 1:5 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

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

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards and samples.

PRECAUTIONS

The Haptoglobin Standard is derived from human serum. The serum was tested at the donor level using an FDA licensed method and found to be non-reactive for anti-HIV-1/2 and Hepatitis B surface antigen. As no testing can offer complete assurance of freedom from infectious agents, this reagent should be handled as if capable of transmitting infection.

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

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the 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.

Serum-Free 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 animal serum are not suitable for use in this assay due to the high potential for hemoglobin contamination.*

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Hemolyzed samples are not suitable for use in this assay.

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

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

Serum and plasma samples require at least a 20,000-fold dilution:

1. Add 20 µL of sample to 480 µL of Calibrator Diluent RD5-67 (diluted 1:5).
2. Add 20 µL of the diluted sample from step one to 480 µL of Calibrator Diluent RD5-67 (diluted 1:5).
3. Complete the 20,000-fold dilution by adding 20 µL of the diluted sample from step two to 620 µL Calibrator Diluent RD5-67 (diluted 1:5).

Saliva samples require at least a 2-fold dilution. A suggested 2-fold dilution is 50 µL of sample + 50 µL of Calibrator Diluent RD5-67 (diluted 1:5).

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *Haptoglobin is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

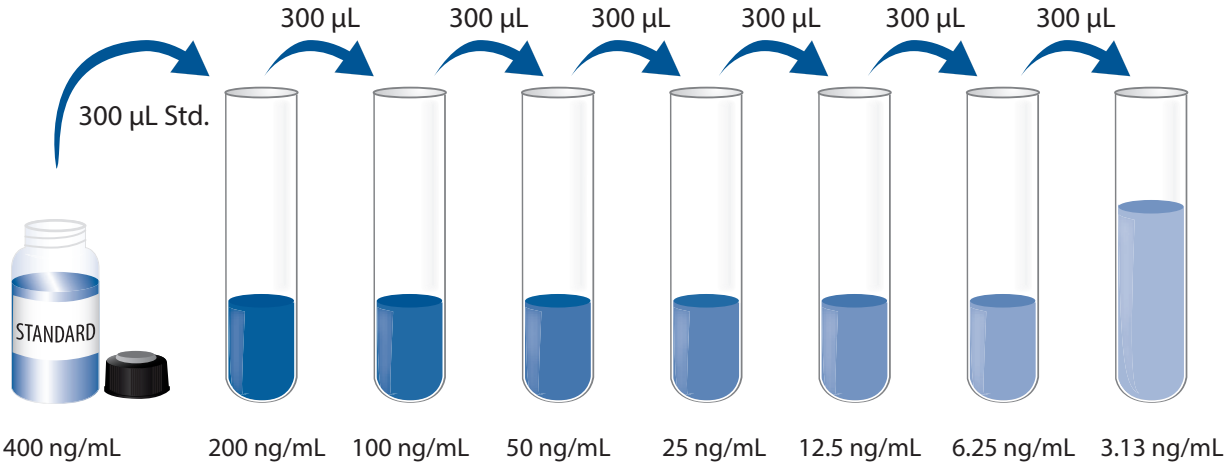
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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.

Calibrator Diluent RD5-67 (diluted 1:5) - Add 20 mL of Calibrator Diluent RD5-67 to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5-67 (diluted 1:5).

Human Haptoglobin Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Haptoglobin Standard with deionized or distilled water. This reconstitution produces a stock solution of 400 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.

Pipette 300 µL of Calibrator Diluent RD5-67 (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 200 ng/mL standard serves as the high standard. Calibrator Diluent RD5-67 (diluted 1:5) serves as the zero standard (0 ng/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: *Haptoglobin is 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 200 µL of Assay Diluent RD1-109 to each well.
4. Add 20 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 Haptoglobin 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 the Sample Preparation section.

CALCULATION OF RESULTS

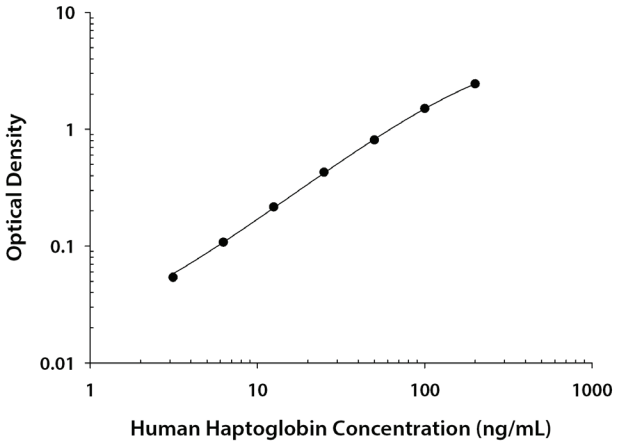
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 Haptoglobin 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	0.011 0.013	0.012	—
3.13	0.065 0.067	0.066	0.054
6.25	0.118 0.122	0.120	0.108
12.5	0.225 0.230	0.228	0.216
25	0.440 0.441	0.441	0.429
50	0.807 0.832	0.820	0.808
100	1.508 1.519	1.514	1.502
200	2.413 2.504	2.459	2.447

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 (ng/mL)	18.7	58.3	116	18.8	57.8	113
Standard deviation	0.690	1.42	3.66	1.40	3.47	6.30
CV (%)	3.7	2.4	3.2	7.4	6.0	5.6

RECOVERY

The recovery of human Haptoglobin spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Serum-free cell culture media (n=4)	101	94-107%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Haptoglobin 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)	Saliva* (n=4)	Urine (n=4)
1:2	Average % of Expected	103	102	106	104	101	107
	Range (%)	96-107	95-107	101-112	102-107	97-106	102-111
1:4	Average % of Expected	103	103	106	107	98	105
	Range (%)	99-107	98-109	98-114	100-114	97-106	97-109
1:8	Average % of Expected	100	106	110	106	100	109
	Range (%)	98-105	101-113	106-118	100-114	96-102	106-117
1:16	Average % of Expected	96	104	108	106	99	110
	Range (%)	93-101	99-111	102-118	96-115	87-106	104-115

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

SENSITIVITY

Twenty-five assays were evaluated and the minimum detectable dose (MDD) of human Haptoglobin ranged from 0.031-0.529 ng/mL. The mean MDD was 0.192 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 highly purified Haptoglobin from human serum.

SAMPLE VALUES

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

Sample Type	Mean (mg/mL)	Range (mg/mL)	Standard Deviation (mg/mL)
Serum (n=36)	0.903	0.159-2.30	0.429
EDTA plasma (n=36)	0.852	0.149-2.81	0.484
Heparin plasma (n=36)	0.839	0.143-2.45	0.453

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Saliva (n=17)	485	10.4-2302	578

Sample Type	Mean of Detectable (ng/mL)	% Detectable	Range (ng/mL)
Urine (n=21)	55.6	71.4	ND-176

ND=Non-detectable

Serum-Free Cell Culture Supernates - Hep G2 human hepatocellular carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum until confluent. Media was then removed by centrifugation and cells were washed 2 times with PBS. Serum-free DMEM was added and cells were cultured an additional 24 hours. An aliquot of the serum-free cell culture supernate was removed, assayed for human Haptoglobin, and measured 36.2 ng/mL.

SPECIFICITY

This assay recognizes natural human Haptoglobin. This assay also recognizes natural human Haptoglobin 1-1 and Haptoglobin 2-2.

The factors listed below were prepared at 5.0 µg/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 5.0 µg/mL in a mid-range recombinant human Haptoglobin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

CD163
Coagulation Factor VII
Coagulation Factor X
Coagulation Factor Xa
Complement Component C1r
Complement Component C1s
Chymotrypsin C
Marapsin
MASP-3
Spinesin
Thrombin

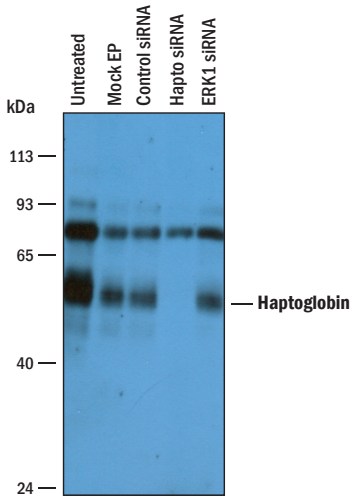
Recombinant mouse:

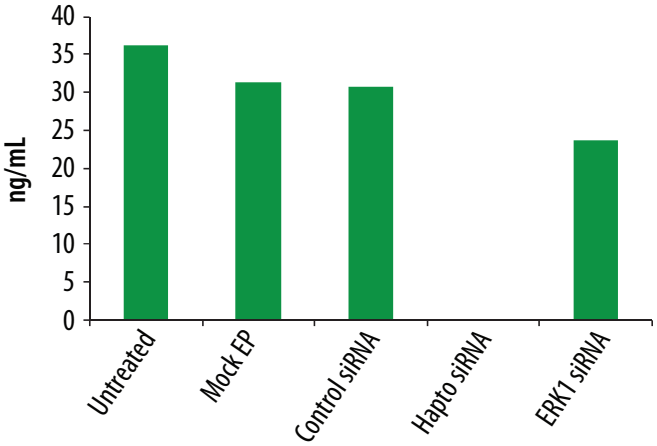
Coagulation Factor VII
Coagulation Factor Xa
Marapsin
Spinesin

Natural proteins:

bovine Coagulation Factor Xa
human Plasminogen
human Prothrombin

Recombinant mouse Haptoglobin cross-reacts approximately 0.855% in this assay.





Hep G2 cells were either left untreated, mock electroporated (Mock EP), or electroporated with the indicated siRNA oligonucleotides: Control against a region of Haptoglobin that does not affect expression, or specific Haptoglobin or ERK1 oligonucleotides. Conditioned media from these Hep G2 samples were resolved under reducing SDS-PAGE conditions, transferred to a PVDF membrane, and immunoblotted with the detection antibody used in this kit. The immunoprecipitation/Western Blot shows direct correlation with the ELISA value for these samples.

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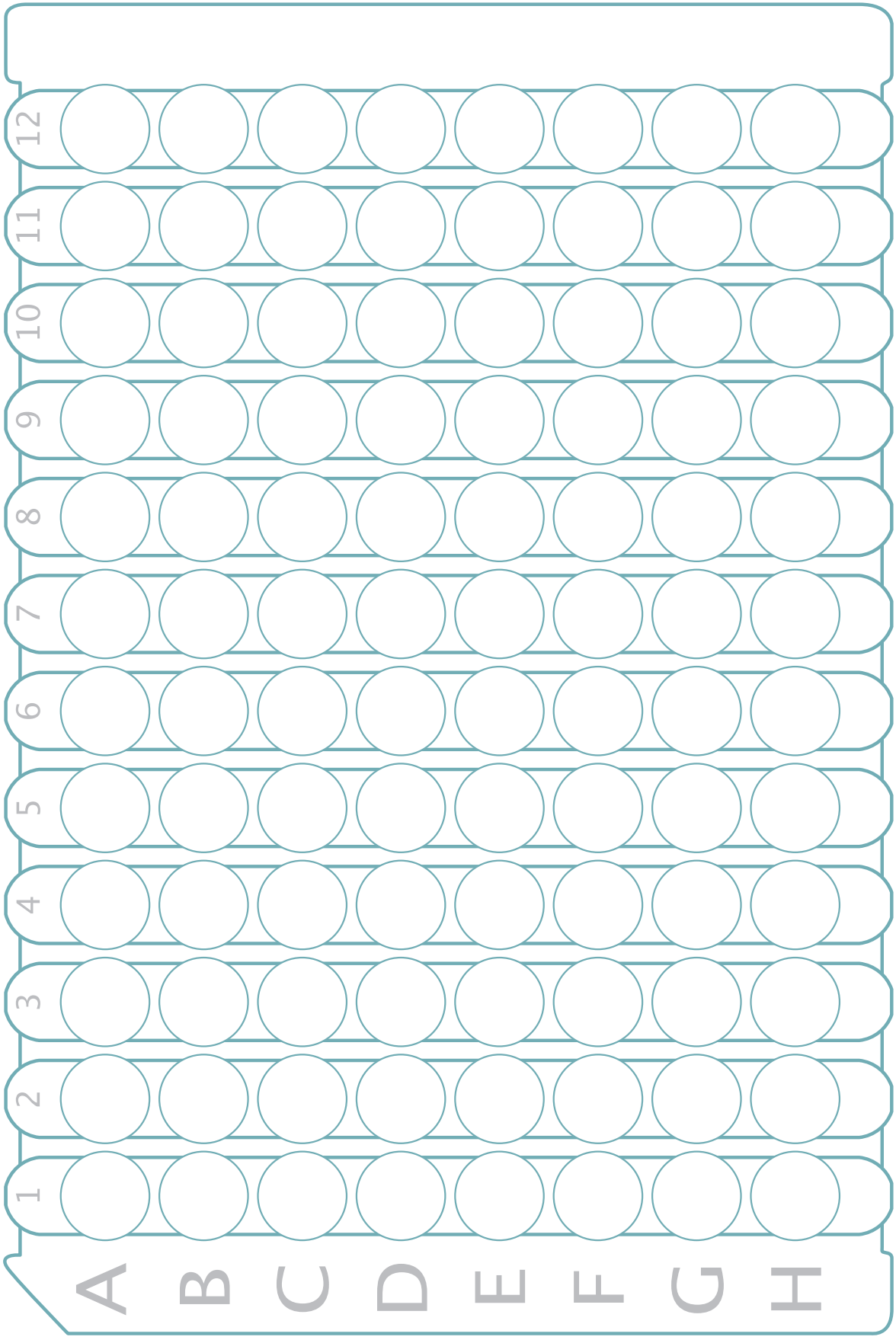
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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