Quantikine® ELISA

Human Relaxin-2 Immunoassay

Catalog Number DRL200

For the quantitative determination of human Relaxin-2 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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INTRODUCTION

Human Relaxin-2, also known as H2 Relaxin, is a peptide hormone best known for its important physiological roles during pregnancy. Relaxin-2 belongs to a structurally-related insulin/relaxin superfamily that contains ten members in humans, including insulin, insulin-like growth factors, insulin-like peptides, relaxins, and relaxin-like factors (1, 2). Relaxin-2 is synthesized and secreted as a preprohormone that is 18 kDa in size and 185 amino acids (aa) in length. Like other family members, it contains a signal peptide and B, C and A domains respectively arranged from N- to C-termini (1-4). Six invariant cysteine residues in the A and B chains form two interdomain disulfide bonds between the B and A chains and one intradomain disulfide bond in the A chain (1, 4). Prohormone convertase removes the intervening C chain to yield a 6 kDa disulfide-linked heterodimer (5). The presence of an Arg-X-X-X-Arg-X-X-Ile motif in the B chain distinguishes relaxins from other insulin/relaxin superfamily members (1, 6). This motif is indispensable for relaxin biological activity and interaction with the relaxin receptor (6, 7). Human Relaxin-2 is found on chromosome 9, at a locus shared with Relaxin-1 (8). Relaxin-2 encodes the predominant circulating form in humans that serves as the functional equivalent to Relaxin-1 in non-primate mammals (1, 6, 9). Although it has bioactivity similar to Relaxin-2, Relaxin-1 is not thought to be the main player during human pregnancy due to its restricted expression pattern and the limited quantities found in circulation (10, 11). An alternately spliced form, incorporating an extra exon in the C domain for Relaxin-2 has been reported, although its significance is unclear (12). Expression of Relaxin-2 appears to be transcriptionally regulated as the promoter of this gene contains response elements for progesterone, glucocorticoid, zinc, mineralocorticoid, $NF_{\kappa}B$, AP-1 and SP-1 (2, 13-16).

The role of relaxin in pregnancy physiology differs considerably between mammals. The often-cited childbirth-related functions of softening the cervix, widening the pubic symphisis and promoting mammary gland development are relaxin-dependent only in some animals, not in humans (1, 2, 17). In humans and other primates, relaxin does play a role in pregnancy, albeit an earlier role of modulating endometrial physiology during implantation (2, 18). In rodents there is a major pre-labor surge in circulating relaxin levels, corresponding to its role in parturition; however, in humans and other primates, the serum levels of relaxin are highest during the first trimester (17). In women, the ovarian *corpus luteum* is the source of circulating Relaxin-2. In non-conception cycles it rises and peaks within 10 days of ovulation, declining with the demise of the *corpus luteum* (2). During conception cycles, Relaxin-2 secretion is maintained in maternal circulation and continues to rise to reach between 1.0-3.5 ng/mL in the first trimester (1, 2, 18). Levels drop approximately 20% at the end of the first trimester before leveling off for the remainder of the pregnancy (19, 20). Local sites of synthesis and action for this hormone are also important. The decidua, placenta, and fetus also synthesize Relaxin-2, which shows increased expression in patients with preterm birth due to premature rupture of the fetal membranes (21). Secretion from primary cultures of endometrial stromal and glandular epithelial cells indicate the endometrium is a souce for Relaxin-2, where it acts as a paracrine factor to stimulate production of several endometrial factors such as prolactin, IGFBP-1, and VEGF (22, 23). Uterine effects of Relaxin-2 include increased weight along with stimulation of endometrial angiogenesis and of cytokine-containing lymphocytes (18). Relaxin also helps maintain endometrial collagen content and matrix integrity by balancing levels of

MMPs and their inhibitor, TIMP-1 (18). While these effects suggest a role for Relaxin-2 in establishing and maintaining early pregnancy, luteal relaxin is not required for successful term pregnancy; patients with premature ovarian failure (and no *corpus luteum* nor circulating relaxin) do have successful pregnancies with an egg donor (24). It should be noted that Relaxin-2 is also present in males and is synthesized by non-reproductive organs such as the kidney, heart, and lungs suggesting a more global contribution for this hormone (1, 17, 25, 26). Studies in rodents and pigs suggest that Relaxin also reduces fibrosis, facilitates wound healing, and protects the heart against ischemia-induced injury (1).

Relaxin-2 initiates its pleiotropic effects through multiple pathways on a variety of cell types. It confers activity by binding to the leucine-rich G protein-coupled receptor LRG7, and with significantly less affinity to LRG8 (27). This leads to an increase in cAMP and activation of PKA (27, 28). LRG7 and LRG8 are 7-pass transmembrane proteins with a relatively large extracellular N-terminus and an intracellular C-terminus (28). They belong to the same receptor subclass as receptors for the pituitary hormones LH and FSH (28). There is also evidence that Relaxin-2 signals through receptor tyrosine kinase signaling pathways in primary uterine and THP-1 monocytic cells; MAPK pathway in endometrial stromal, THP-1, and coronary artery smooth muscle cells; ERK1/2 in HUVEC and HeLa epithelial cells; nitric oxide/cGMP in basophils, neutrophils, and breast cancer cells; and a novel phosphotyrosine-dependent pathway in THP-1 cells (29-37).

The Quantikine® Human Relaxin-2 Immunoassay is a 4.5 hour solid phase ELISA designed to measure human Relaxin-2 in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant human Relaxin-2 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Relaxin-2 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 Relaxin-2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Relaxin-2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Relaxin-2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Relaxin-2 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 Relaxin-2 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 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.

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 Relaxin-2 Microplate	893570	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Relaxin-2.	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 Relaxin-2 Conjugate	893571	21 mL of a polyclonal antibody specific for human Relaxin-2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Relaxin-2 Standard	893572	Recombinant human Relaxin-2 in a buffered protein solution with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-19	895467	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-18	895335	21 mL of a buffered protein base with preservatives. *For cell culture supernate samples.*	
Calibrator Diluent RD6-6	895177	21 mL of a buffered protein base with preservatives. *For serum/plasma 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.
- Test tubes for dilution of standards.
- Human Relaxin-2 Controls (optional; R&D Systems®, Catalog # QC153).

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 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.*

REAGENT PREPARATION

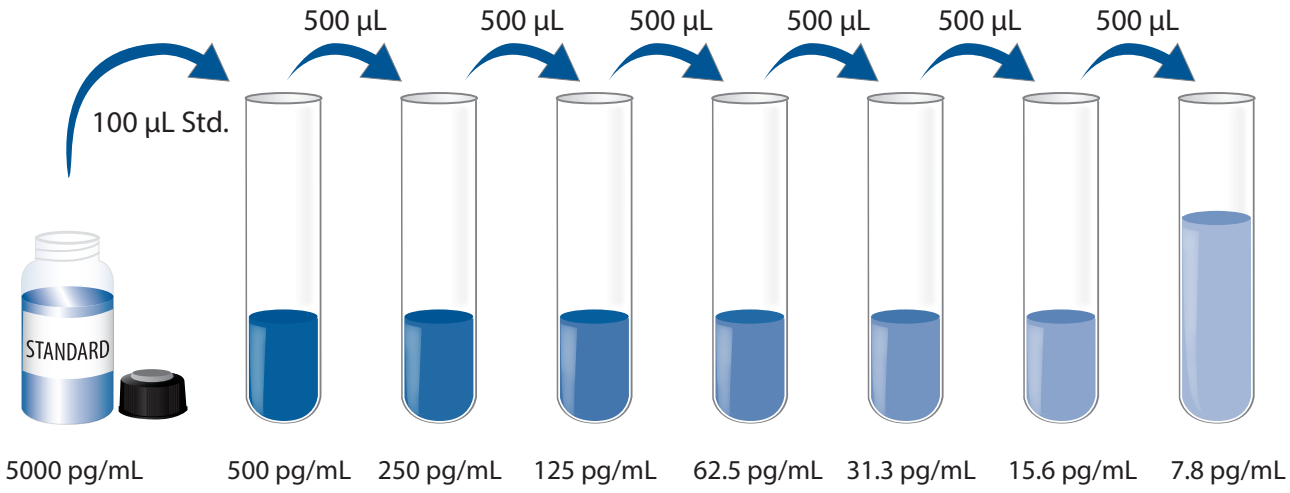
Bring all reagents to room temperature before use.

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.

Human Relaxin-2 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Relaxin-2 Standard with deionized or distilled water. This reconstitution produces a stock solution of 5000 pg/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 900 µL of Calibrator Diluent RD5-18 (*for cell culture supernate samples*) or Calibrator Diluent RD6-6 (*for serum/plasma samples*) into the 500 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 500 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 samples, controls, and standards 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 RD1-19 to each well.
4. Add 50 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. 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 Relaxin-2 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
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.

CALCULATION OF RESULTS

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

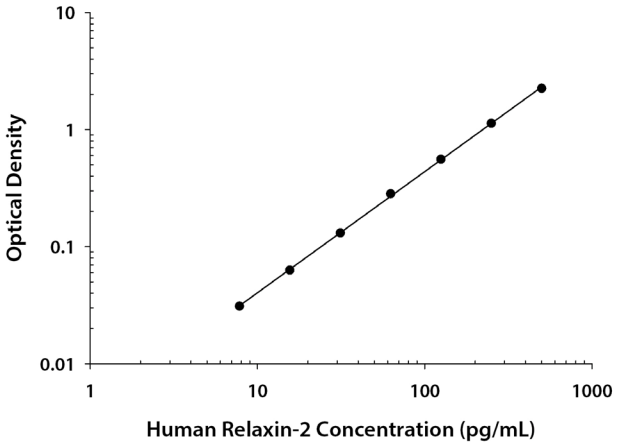
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human Relaxin-2 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

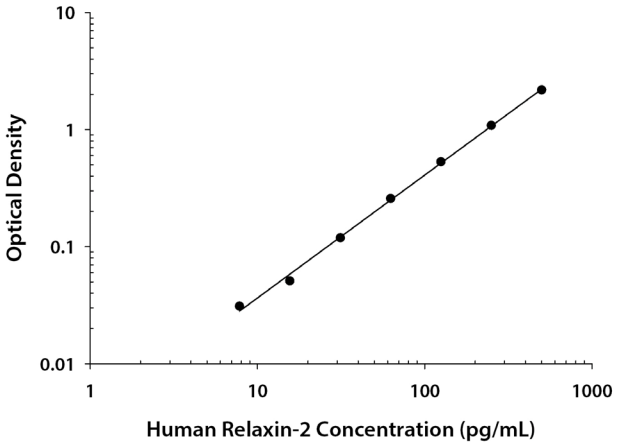
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.009 0.011	0.010	—
7.8	0.040 0.041	0.041	0.031
15.6	0.071 0.074	0.073	0.063
31.3	0.139 0.142	0.141	0.131
62.5	0.281 0.305	0.293	0.283
125	0.561 0.574	0.568	0.558
250	1.112 1.169	1.141	1.131
500	2.236 2.281	2.259	2.249

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.009 0.009	0.009	—
7.8	0.039 0.040	0.040	0.031
15.6	0.059 0.060	0.060	0.051
31.3	0.122 0.133	0.128	0.119
62.5	0.265 0.269	0.267	0.258
125	0.532 0.547	0.540	0.531
250	1.077 1.110	1.094	1.085
500	2.181 2.202	2.192	2.183

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 separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	41	41	41
Mean (pg/mL)	35.1	96.0	194	34.9	98.9	197
Standard deviation	1.27	2.44	4.01	3.41	5.90	11.7
CV (%)	3.6	2.5	2.1	9.8	6.0	5.9

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	42	42	42
Mean (pg/mL)	34.4	99.6	206	40.8	112	220
Standard deviation	1.60	2.47	4.75	4.16	7.02	12.1
CV (%)	4.7	2.5	2.3	10.2	6.3	5.5

RECOVERY

The recovery of human Relaxin-2 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	95	89-102%
Serum (n=4)	101	92-107%
EDTA plasma (n=4)	98	93-106%
Heparin plasma (n=4)	97	91-104%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Relaxin-2 were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	102	102	105	106
	Range (%)	101-103	100-103	103-106	102-109
1:4	Average % of Expected	102	101	111	107
	Range (%)	101-104	98-104	109-113	104-111
1:8	Average % of Expected	102	100	110	108
	Range (%)	100-104	95-106	105-118	103-115
1:16	Average % of Expected	103	98	107	108
	Range (%)	98-107	91-102	104-110	100-114

SENSITIVITY

Eighty assays were evaluated and the minimum detectable dose (MDD) of human Relaxin-2 ranged from 0.26-4.57 pg/mL. The mean MDD was 1.00 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 Relaxin-2.

SAMPLE VALUES

Serum/Plasma - Thirty-five serum and plasma samples from apparently healthy volunteers were evaluated for the presence of human Relaxin-2 in this assay. All samples measured below the lowest standard, 7.8 pg/mL. No medical histories were available for the donors used in this study.

Cell Culture Supernates - Human peripheral blood lymphocytes (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed on days 1 and 5 and assayed for levels of human Relaxin-2. No detectable levels were observed.

SPECIFICITY

This assay recognizes natural and recombinant human Relaxin-2.

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 Relaxin-2 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

IGF-I
IGF-II
Insulin (aa 25 - 110)
Insulin (pro) K (aa 25 - 110) N His
Insulin-like 3
Relaxin-1
Relaxin-3

Other recombinants:

canine Relaxin-2
mouse Relaxin-2
porcine Relaxin-2
rat Relaxin-2

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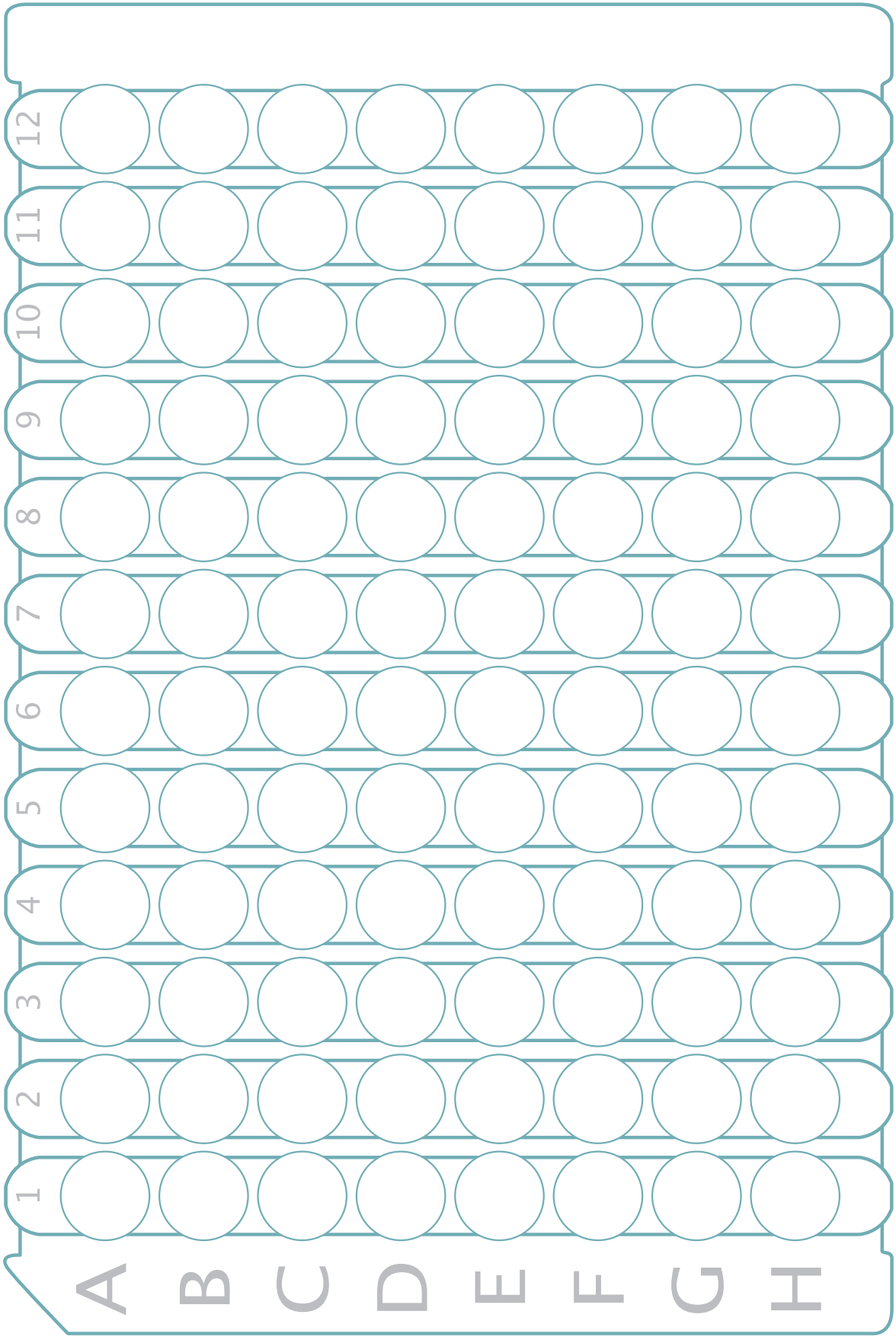
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES