Quantikine™ ELISA

BMP-2 Immunoassay

Catalog Number DBP200
SBP200
PDBP200

For the quantitative determination of Bone Morphogenetic Protein 2 (BMP-2) concentrations in bone tissue extracts and cell culture supernates.

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

Bone morphogenetic protein-2 (BMP-2, previously known as BMP-2A) is a member of the transforming growth factor beta (TGF-β) superfamily, based on amino acid (aa) sequence homology (1). BMPs were originally identified as protein regulators of cartilage and bone formation. They have also been implicated in embryogenesis and morphogenesis of various tissues and organs.They can regulate growth, differentiation, chemotaxis and apoptosis of a variety of cell types, including mesenchymal, epithelial, hematopoietic and neuronal cells. BMP-2 has pleiotropic functions including organogenesis, bone formation and regeneration, and regulation of pattern formation in the developing limb bud (1-7). Recombinant human BMP-2 has been shown to possess potent ectopic bone forming activity in a variety of experimental systems (6, 7).

Each BMP is synthesized as a precursor peptide, processed to a mature form, and subsequently secreted as a dimer. Although homodimers are considered the standard form, there are natural heterodimers with equal, if not increased, bioactivity (8, 9). BMP-2 is a 396 amino acid (aa) glycosylated polypeptide composed of a 19 aa signal sequence, a 263 aa pro-region, and a 114 aa mature segment (1). The mature region of BMP-2 has seven cysteines and one N-linked glycosylation site. Although the predicted mass of BMP-2 is 14 kDa, the mature segment is actually 18 kDa and is assumed to be glycosylated. The mature regions of human, mouse and rat BMP-2 are identical. With respect to other BMPs, BMP-2 and BMP-4 are 92% identical at the aa level and are therefore considered a subgroup within the BMP family (5). The human gene for BMP-2 maps to chromosome 20p12 (10, 11).

BMPs signal via different hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors (for reviews, see references 12 and 13). BMP-2 receptors include the type I receptors, ALK-6/BMPR-IB, ALK-2/Act RI and ALK-3/BMPR-IA, and the type II receptors, BMP RII and Act RIIB (14-18). Endoglin, an accessory protein that interacts with the signaling receptor complex of TGF-β superfamily members, can bind BMP-2 through interaction with the type I receptors, ALK-3/BMPR-IA and ALK-6/BMPR-IB (19). Signals from activated BMP receptors are directly transduced to the cell nucleus by Smad proteins that then become incorporated into transcriptional complexes (for a review, see reference 20). The Smad1 pathway, for example, is involved in BMP-2 signaling (21, 22).

BMPs are important signaling molecules for embryonic development processes. During endochondral development, cartilage and bone differentiation involve a series of events that are directly influenced by BMPs. BMP-2, for example, obviously plays a critical role for development in mice, as BMP-2 gene knockout by homologous recombination results in embryonic lethality (2). Endochondral bone formation is not only necessary for limb formation in embryogenesis, but is also required for longitudinal bone growth in postnatal life and bone regeneration following injury. BMP-2 is expressed in the growth plate and regulates growth plate chondrogenesis by inducing chondrocyte proliferation and hypertrophy (23, 24). In addition to promoting bone formation during embryonic development, BMP-2 is also involved in dorsal-ventral pattern formation. BMP-2 influences patterning within mouse, zebrafish and *Xenopus* embryos (25-27).

The Quantikine™ Human BMP-2 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure BMP-2 in bone tissue extracts and cell culture supernates. It contains CHO cell-expressed recombinant human BMP-2 and has been shown to accurately quantitate the recombinant factor. This kit does not recognize *E. coli*-expressed BMP-2. Results obtained using natural mouse BMP-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 naturally occurring BMP-2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for BMP-2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any BMP-2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for BMP-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 BMP-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 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 # DBP200	CATALOG # SBP200	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
BMP-2 Microplate	892141	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for BMP-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.*
BMP-2 Standard	892143	1 vial	6 vials	Recombinant human BMP-2 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and freeze at ≤ -20 °C for up to 1 month.*
BMP-2 Conjugate	892142	1 vial	6 vials	21 mL/vial of monoclonal antibody specific for BMP-2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-19	895467	1 vial	6 vials	11 mL/vial of a buffered protein base with preservatives.	
Calibrator Diluent RD5P	895151	1 vial	6 vials	21 mL/vial of a buffered protein base with preservatives. *Use diluted 1:10 in this assay.*	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial 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/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

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

DBP200 contains sufficient materials to run an ELISA on one 96 well plate.
SBP200 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PDBP200). Refer to the PharmPak Contents section for specific vial counts.

PHARMPAK CONTENTS

Each PharmPak contains reagents sufficient for the assay of 50 microplates (96 wells/plate). The package inserts supplied are the same as those supplied in the single kit packs and because of this, a few minor differences related to the number of reagents and their container sizes should be noted.

- Sufficient material is supplied to perform at least 50 standard curves; reuse of each vial may be required. The number of vials, and the number of standard curves obtained per vial will vary with the analyte.
- Wash Buffer 25X Concentrate is bulk packed in 125 mL bottles containing 100 mL.
  Note: *Additional wash buffer is available for purchase (R&D Systems®, Catalog # WA126).*

The reagents provided in this PharmPak are detailed below.

PART	PART #	QUANTITY
BMP-2 Microplate	892141	50 plates
BMP-2 Standard	892143	25 vials
BMP-2 Conjugate	892142	50 vials
Assay Diluent RD1-19	895467	50 vials
Calibrator Diluent RD5P	895151	50 vials
Wash Buffer Concentrate	895126	50 vials
Color Reagent A	895000	50 vials
Color Reagent B	895001	50 vials
Stop Solution	895032	9 bottles
Plate sealers	N/A	100 sheets
Package inserts	750724	2 booklets

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
- 250 mL and 500 mL graduated cylinders
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 rpm ± 50 rpm
- **Polypropylene** test tubes for dilution of standards
- BMP-2 Controls (optional; R&D Systems®, Catalog # QC49).

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. Use polypropylene tubes/vials for sample handling and storage

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Bone - Extract demineralized bone samples in 4 M Guanidine-HCl and protease inhibitors (28, 29). Dissolve the final sample in 2 M Guanidine-HCl.

Note: *Extractions can also be done in urea (29, 30).*

Bone extract samples must be diluted in Calibrator Diluent RD5P (diluted 1:10)* prior to assay so that the final concentration of Guanidine-HCl is ≤ 0.06 M, or the final concentration of Urea is ≤ 0.25 M.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

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

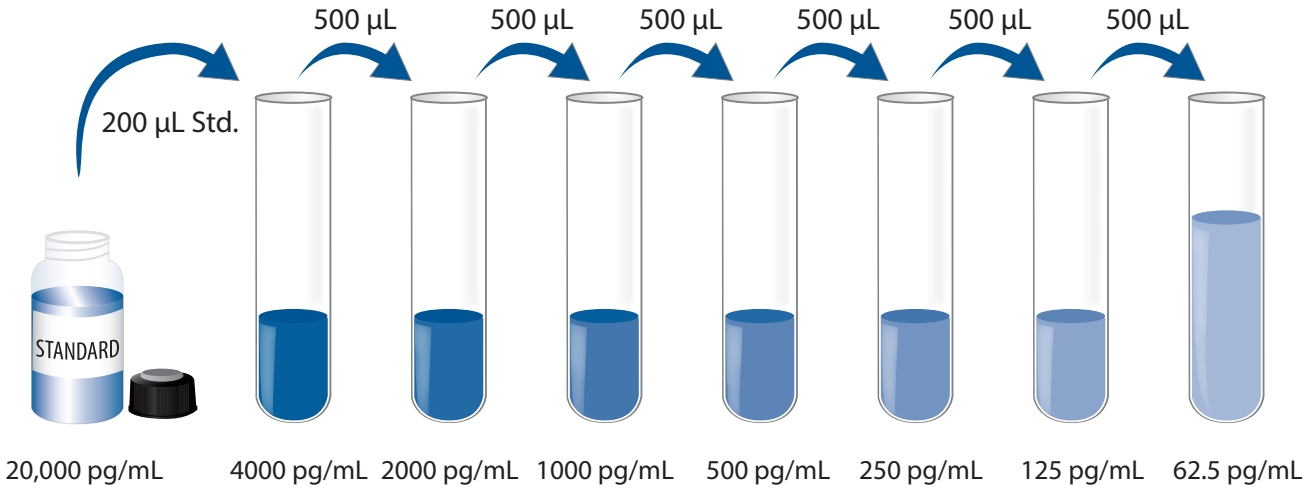
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5P (diluted 1:10) - Add 20 mL of Calibrator Diluent RD5P to 180 mL of deionized or distilled water to prepare 200 mL of Calibrator Diluent RD5P (diluted 1:10). Mix for 15 minutes prior to use.

BMP-2 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the BMP-2 Standard with deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/mL. Mix the standard by inversion or brief vortex for 5-10 seconds to ensure complete reconstitution and then allow the standard to sit for a minimum of 15 minutes prior to making dilutions.

Note: *Do not use rocker or extended vortex.*

Use polypropylene tubes. Pipette 800 µL of Calibrator Diluent RD5P (diluted 1:10) into the 4000 pg/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 4000 pg/mL standard serves as the high standard. Calibrator Diluent RD5P (diluted 1:10) serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent 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 on a horizontal orbital microplate shaker (0.12" orbit) set at 500 rpm ± 50 rpm. A plate layout is provided to record the 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 BMP-2 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.

*Bone samples require extraction and dilution. See Sample Collection Section.

CALCULATION OF RESULTS

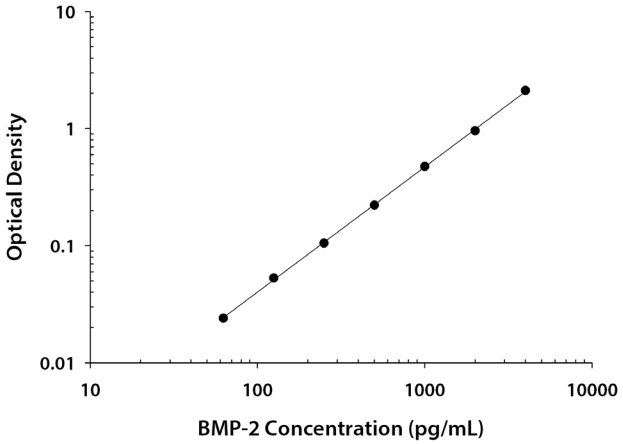
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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

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	0.035 0.036	0.036	—
62.5	0.060 0.060	0.060	0.024
125	0.088 0.089	0.089	0.053
250	0.140 0.142	0.141	0.105
500	0.258 0.258	0.258	0.222
1000	0.504 0.515	0.510	0.474
2000	0.992 0.996	0.994	0.958
4000	2.069 2.233	2.151	2.115

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 (pg/mL)	323	1010	2313	344	1043	2213
Standard deviation	8.3	28.3	56.0	25.2	55.4	140.2
CV (%)	2.6	2.8	2.4	7.3	5.3	6.3

RECOVERY

The recovery of recombinant human BMP-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)	102	94-110%
2 M Guanidine-HCl* (n=1)	98	94-101%
2 M Urea* (n=1)	101	92-106%

*Samples were diluted prior to assay.

LINEARITY

The following samples were spiked with BMP-2 and then diluted with calibrator diluent. The samples were serially diluted and tested to assess the linearity of the assay.

		Cell culture media (n=4)	2 M Guanidine HCl* (n=1)	2 M Urea (n=1)
1:2	Average % of Expected	103	100	103
	Range (%)	97-109	———	———
1:4	Average % of Expected	103	103	106
	Range (%)	98-110	———	———
1:8	Average % of Expected	109	112	114
	Range (%)	104-113	———	———
1:16	Average % of Expected	108	114	113
	Range (%)	102-112	———	———

*Samples were diluted prior to assay.

SENSITIVITY

Thirty-five assays were evaluated and the minimum detectable dose (MDD) of BMP-2 ranged from 4.3-29 pg/mL. The mean MDD was 11 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 CHO cell-expressed recombinant human BMP-2 produced at R&D Systems. This assay immunoassay does not recognize *E. coli*-expressed BMP-2.

The NIBSC/WHO Reference Reagent recombinant human BMP-2 Standard 93/574 was evaluated in this assay. The dose response curve of the NIBSC Standard 93/574 parallels the Quantikine™ standard curve. To convert sample values obtained with the Quantikine BMP-2 kit to approximate NIBSC units, use the equation below.

NIBSC (93/574) approximate value (U/mL) = 0.0006 x Quantikine BMP-2 value (pg/mL)

SAMPLE VALUES

Cell Culture Supernates - Cell culture supernates from the following unstimulated cell lines were tested for natural BMP-2. No BMP-2 was detected.

Cell Lines	Type	Growth Conditions
ATDC5	Mouse chondrogenic	1:1 mixture of F-12 and DMEM + 5% FBS, L-glutamine, penicillin, and streptomycin
MCF-7	Human breast cancer	1:1 mixture of high glucose DMEM and F-12 + 10% FBS, L-glutamine, penicillin, and streptomycin
MDA-MB-453	Human breast cancer	RPMI + 10% FBS, L-glutamine, penicillin, and streptomycin
ST-2	Mouse-bone marrow stroma-cell derived	RPMI + 10% FBS, L-glutamine, penicillin, and streptomycin
U2OS	Human osteosarcoma	Grown to confluency in McCoy's 5a media + 15% FBS, L-glutamine, penicillin, and streptomycin

SPECIFICITY

This assay recognizes mammalian-expressed recombinant and natural BMP-2. This assay does not recognize *E. coli*-expressed BMP-2.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5P (diluted 1:10) and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant BMP-2 standard were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Activin A
Activin RIA
Activin RIIA
Activin RIIB
BMP-2/BMP-7 Heterodimer
BMP-5
BMP-6
BMP-7
BMPR-IB
Follistatin 288
Follistatin 300
Follistatin 315
Inhibin A
Inhibin B
LAP
TGF-α
TGF-β sRII
TGF-β sRIII
TGF-β1
TGF-β1.2
TGF-β2
TGF-β3

Recombinant mouse:

BMPR-IB
Follistatin

Other recombinants:

rat Agrin
porcine TGF-β2
amphibian TGF-β5

Natural proteins:

human TGF-β1
porcine TGF-β1

Recombinant human BMPR-IA and recombinant mouse BMPR-IA were found to interfere at concentrations > 10 ng/mL. Recombinant mouse Noggin was found to interfere at concentrations > 5 ng/mL.

Cross-reactivity was observed to be 1.2% with 50 ng/mL of recombinant human BMP-4.

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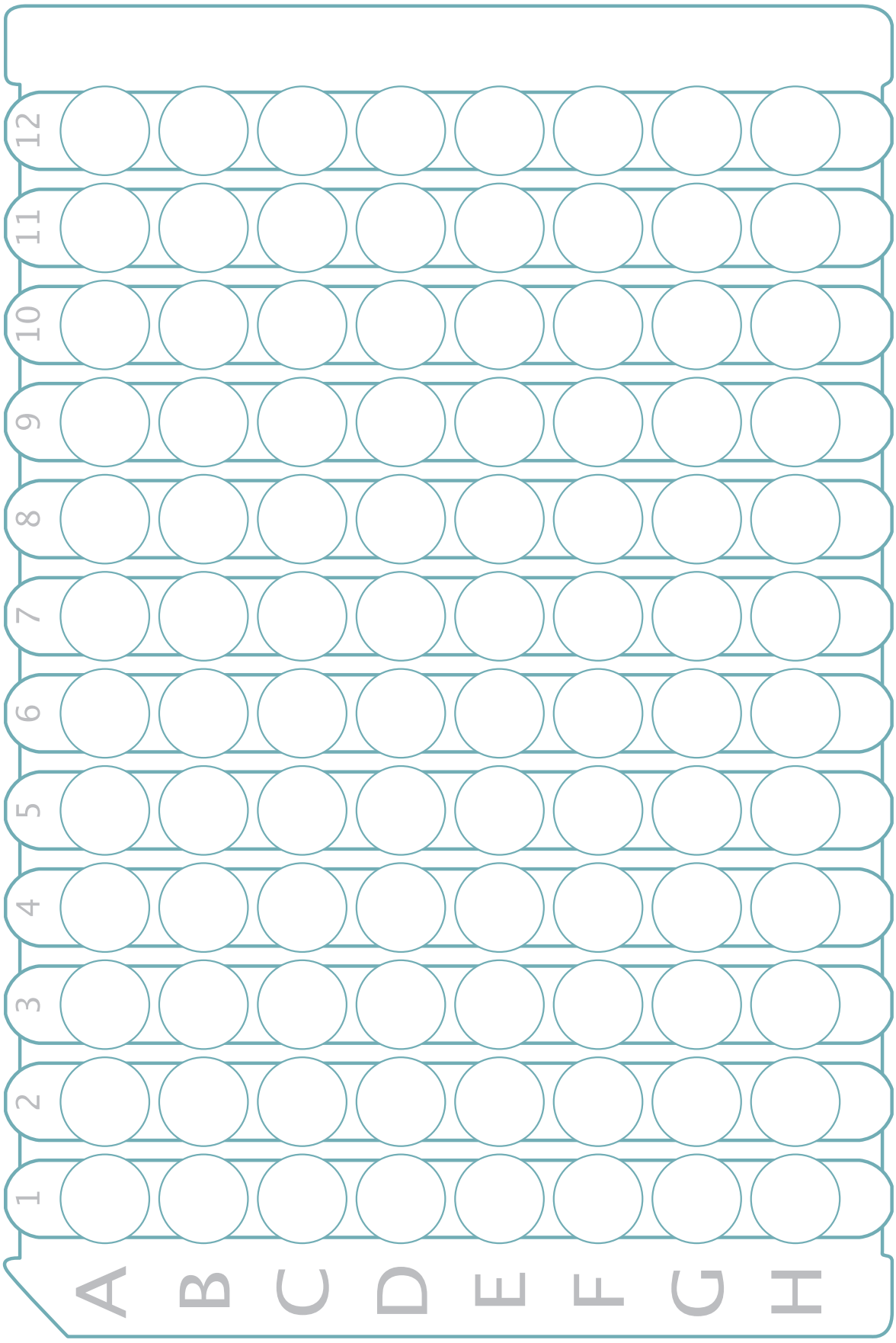
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES