Parameter™

Cortisol Assay

Catalog Number KGE008B
SKGE008B
PKGE008B

For the quantitative determination of Cortisol concentrations in 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

Cortisol, also known as Hydrocortisone or Compound F, is the primary human glucocorticoid synthesized by the adrenal cortex. Cortisol has multiple roles, but may be best known for its effects on metabolism and immune system function. It is a metabolite of cholesterol and a precursor for other steroid hormone classes including mineralcorticoids, estrogens, androgens, and progestins (1).

Cortisol levels are controlled by the hypothalamic-pituitary-adrenal (HPA) axis (2). It is secreted by the adrenal cortex in response to adrenocorticotropic hormone (ACTH) released by the anterior pituitary. ACTH secretion is stimulated by peptides such as Corticotropin-releasing Hormone (CRH) and Arginine Vasopressin. A negative feedback loop exists whereby Cortisol suppresses the release of CRH. Cortisol is secreted in pulsatile fashion, and levels are generally highest in the morning just after waking and lowest in the evening (3-5). Approximately 90 percent of Cortisol is associated with binding proteins, primarily Corticosteroid-binding Globulin (CBG/Transcortin) (6). The free form likely mediates most of the physiological effects of Cortisol, although there is some evidence that the CBG-bound form may act as a biological mediator as well (7, 8).

Free Cortisol crosses the cell membrane and enters the cytosol where it binds the widely expressed Glucocorticoid Receptor (GR) (9). Although only a single GR gene has been identified, at least two alternatively spliced isoforms (α and β) exist and potentially have different expression patterns and activities (10). Classically, the hormone/receptor complex enters the nucleus as a dimer where it regulates transcription through binding specific DNA sequences termed Glucocorticoid Response Elements (GRE) (11, 12). In addition to the GR, Cortisol exhibits affinity for the Mineralcorticoid Receptor (MR), the classic receptor for Aldosterone (13). The enzymes 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and 11β-HSD2 regulate the interconversion of Cortisol and biologically inactive Cortisone (14). Therefore, certain MR/11β-HSD-expressing tissues such as the kidney and colon can suppress Cortisol activity and enhance MR selectivity for Aldosterone (15, 16).

Glucocorticoids have a wide range of physiological functions. As the name implies, they are involved in glucose regulation. They increase blood glucose via several different mechanisms that include stimulating gluconeogenesis and insulin resistance (17-20). They also have roles in immune system function and act as prominent immunosuppressive agents (21). Other activities regulated by glucocorticoids include cardiovascular function, cognitive processing, mood, and development (4, 22-27). Cushing's disease is characterized by Cortisol overproduction and is accompanied by a range of effects on metabolism and organ function including hypertension, obesity, muscle wasting, and diabetes (28).

R&D Systems® Cortisol Immunoassay is a 2.5 hour competitive enzyme immunoassay designed to measure Cortisol in cell culture supernates, serum, plasma, saliva, and urine.

PRINCIPLE OF THE ASSAY

This assay is based on the competitive binding technique in which Cortisol present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled Cortisol for sites on a mouse monoclonal antibody. During the incubation, the monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The color development is stopped and the absorbance is read at 450 nm. The intensity of the color is inversely proportional to the concentration of Cortisol in the sample.

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.
- **Samples containing mouse or rat IgG may interfere with this assay.**

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.
- Pre-rinse the pipette tip when pipetting.
- Pipette standards and samples to the bottom of the wells. Add all other reagents to the side of the wells to avoid contamination.
- 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. Gently tap the side of the plate to ensure thorough mixing.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	CATALOG # KGE008B	CATALOG # SKGE008B	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Goat Anti-mouse Microplate	892575	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a goat anti-mouse polyclonal antibody.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.*
Cortisol Conjugate	893053	1 vial	6 vials	6 mL/vial of Cortisol conjugated to horseradish peroxidase with red dye and preservatives.	May be stored for up to 1 month at 2-8 °C.*
Cortisol Standard	893055	1 vial	6 vials	Cortisol in buffer with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Primary Antibody Solution	893054	1 vial	6 vials	6 mL/vial of mouse monoclonal antibody to Cortisol in buffer with blue dye and preservatives.	
Calibrator Diluent RD5-43	895903	2 vials	12 vials	21 mL/vial of a buffered protein base with preservatives.	
Pretreatment E	895996	1 vial	6 vials	11 mL of 0.6 N Trichloroacetic acid	
Pretreatment F	895997	1 vial	6 vials	6 mL of buffer with preservatives.	
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	4 adhesive strips.	

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

KGE008B contains sufficient materials to run an ELISA on one 96 well plate.
SKGE008B (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PKGE008B). 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.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Collection device for saliva samples (Salivette® or equivalent).
- Test tubes for dilution of standards and samples.
- Cortisol Controls (optional; R&D Systems®, Catalog # QC68).

PRECAUTIONS

Pretreatment E contains trichloroacetic acid which is corrosive and causes severe skin burns and eye damage. Do not breathe fumes or mist. Avoid release to the environment. It is very toxic to aquatic life with long lasting effects.

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

Care should be taken when handling the Standard because of the known and unknown effects of steroids.

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.

Samples containing mouse or rat IgG may interfere with this assay.

Cell Culture Supernates - Remove particulates by centrifugation. 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.*

Saliva - Collect saliva using a collection device such as a Salivette or equivalent. 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, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Cortisol shows diurnal variation. It is suggested that all samples in a study be collected at the same time of the day.*

SAMPLE PREPARATION

Cell culture supernate samples require a 10-fold dilution. A suggested 10-fold dilution is 30 µL of sample + 270 µL of Calibrator Diluent RD5-43.

Serum (pretreated), plasma, and urine samples require a 20-fold dilution. A suggested 20-fold dilution is 20 µL of sample + 380 µL of Calibrator Diluent RD5-43.

Saliva samples require a 5-fold dilution. A suggested 5-fold dilution is 50 µL of sample + 200 µL of Calibrator Diluent RD5-43.

SAMPLE PRETREATMENT

Note: ***For serum and plasma samples only.*** *Sample pretreatment removes potentially interfering proteins and protein-bound Cortisol.*

1. Add 200 µL of serum or plasma and 200 µL Pretreatment E to a microcentrifuge tube. The tubes will have a precipitate. Mix well.
2. Incubate for 15 minutes at room temperature.
3. Centrifuge at ≥ 12,000 x g for 4 minutes.
4. Carefully remove and retain the supernate.
5. Add 100 µL of Pretreatment F to 200 µL of the retained supernate. Mix well.
6. The pretreatment step results in a dilution factor of 3. Pretreated serum or plasma samples require an additional 20-fold dilution in Calibrator Diluent RD5-43. The concentration read off the standard curve must be multiplied by the final dilution factor, 60.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

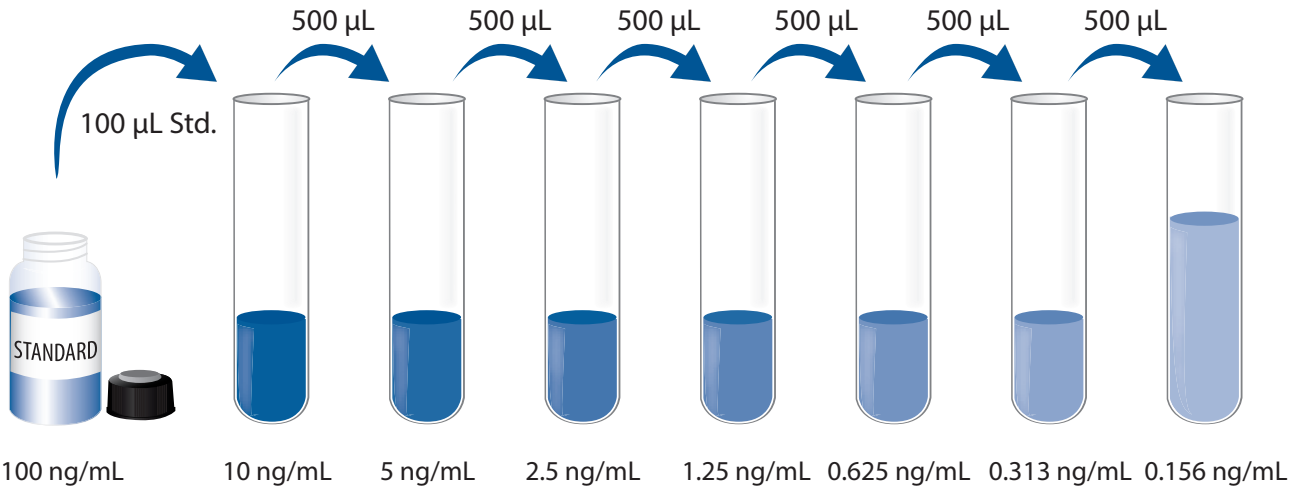
Note: *Cortisol is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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 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.

Cortisol Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Cortisol Standard with deionized or distilled water. This reconstitution produces a stock solution of 100 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 900 µL of Calibrator Diluent RD5-43 into the 10 ng/mL tube. Pipette 500 µL into the remaining tubes. Use the 100 ng/mL standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 10 ng/mL standard serves as the high standard. Calibrator Diluent RD5-43 serves as the zero standard (B_0) (0 ng/mL). **Use diluted standards within 60 minutes of preparation.**



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.

Note: *Cortisol 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 150 µL of Calibrator Diluent RD5-43 into the non-specific binding (NSB) wells.
4. Add 100 µL of Calibrator Diluent RD5-43 to the zero standard (B_0) wells.
5. Add 100 µL of standard or sample* to the appropriate wells. A plate layout is provided to record standards and samples assayed.
6. Add 50 µL of Cortisol Conjugate to all wells. Wells will now be red in color.
7. Add 50 µL of the Primary Antibody Solution to each well **(excluding the NSB wells).** Cover with the adhesive strip provided. All wells will now be violet in color except the NSB wells, which will be red in color.
8. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
9. 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 for 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.
10. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
11. 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 well is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
12. 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 pretreatment or dilution. See Sample Pretreatment and Sample Preparation sections.

CALCULATION OF RESULTS

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

Create a standard curve by reducing the data using computer software capable of generating a four paramater logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on a linear y-axis against the concentration on a logarithmic x-axis and draw the best fit curve through the points on the graph. Do not include the B_0 in the standard curve.

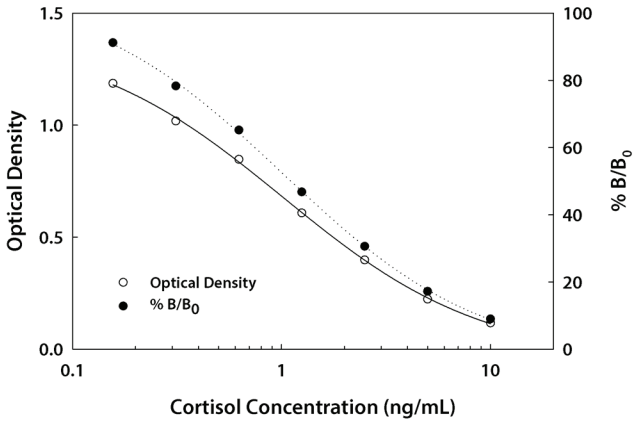
If desired, % B/B_0 can be calculated by dividing the corrected O.D. for each standard or sample by the corrected B_0 O.D. and multiplying by 100.

Calculate the concentration of Cortisol corresponding to the mean absorbance from the standard curve.

Since 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.



(ng/mL)	O.D.	Average	Corrected	% B/B_0
NSB	0.010 0.010	0.010	—	—
0 (B_0)	1.298 1.302	1.300	1.290	—
0.156	1.166 1.225	1.196	1.186	91.2
0.313	1.008 1.047	1.028	1.018	78.3
0.625	0.851 0.862	0.857	0.847	65.2
1.25	0.618 0.618	0.618	0.608	46.8
2.5	0.403 0.413	0.408	0.398	30.6
5	0.231 0.234	0.233	0.223	17.2
10	0.124 0.129	0.127	0.117	9.0

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)	1.30	4.50	6.51	1.11	3.42	5.54
Standard deviation	0.120	0.241	0.411	0.235	0.319	0.576
CV (%)	9.2	5.4	6.3	21.2	9.3	10.4

RECOVERY

The recovery of Cortisol spiked to levels throughout the range of the assay in various matrices was evaluated. Samples were diluted prior to assay.

Sample Type	Average % Recovery	Range %
Cell culture media (n=4)	103	87-113%
Serum* (n=4)	95	89-100%
EDTA plasma* (n=4)	97	94-103%
Heparin plasma* (n=4)	98	92-107%
Urine (n=4)	92	78-113%
Saliva (n=4)	101	80-118%

*Samples were pretreated and/or diluted prior to assay as described in the Sample Pretreatment and Preparation sections.

LINEARITY

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

		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	97	102	102	103	103	107
	Range (%)	90-105	97-109	99-107	93-114	99-108	101-114
1:4	Average % of Expected	95	97	104	101	105	110
	Range (%)	82-108	76-116	93-117	92-109	99-111	101-119
1:8	Average % of Expected	98	106	109	103	103	111
	Range (%)	79-116	95-115	93-120	100-108	86-119	105-119

*Samples were pretreated and/or diluted prior to assay as described in the Sample Pretreatment and Preparation sections.

SENSITIVITY

Thirty-one assays were evaluated and the minimum detectable dose (MDD) of Cortisol ranged from 0.030-0.111 ng/mL. The mean MDD was 0.071 ng/mL.

The MDD was determined by subtracting two standard deviations from the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine - Samples from apparently healthy volunteers were evaluated for the presence of Cortisol 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=34)	90.6	38.2-213	42.1
EDTA plasma n=34)	85.8	32.4-201	40.0
Heparin plasma (n=34)	85.0	35.3-186	37.1
Saliva (n=9)	1.75	0.76-2.94	0.70
Urine (n=13)	56.5	20.8-153	37.4

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

SPECIFICITY

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 Cortisol control were assayed for interference. No significant interference was observed.
Cross-reactivity is listed below.

Compound	% Cross-reactivity
Prednisolone	4.4%
Reichstein's Substance S	3.4%
Progesterone	1.7%
Cortisone	0.2%
4-Androstene-3,17dione	< 0.1%
Corticosterone	< 0.1%
Deoxycorticosterone	< 0.1%
Estradiol	< 0.1%
Prednisone	< 0.1%

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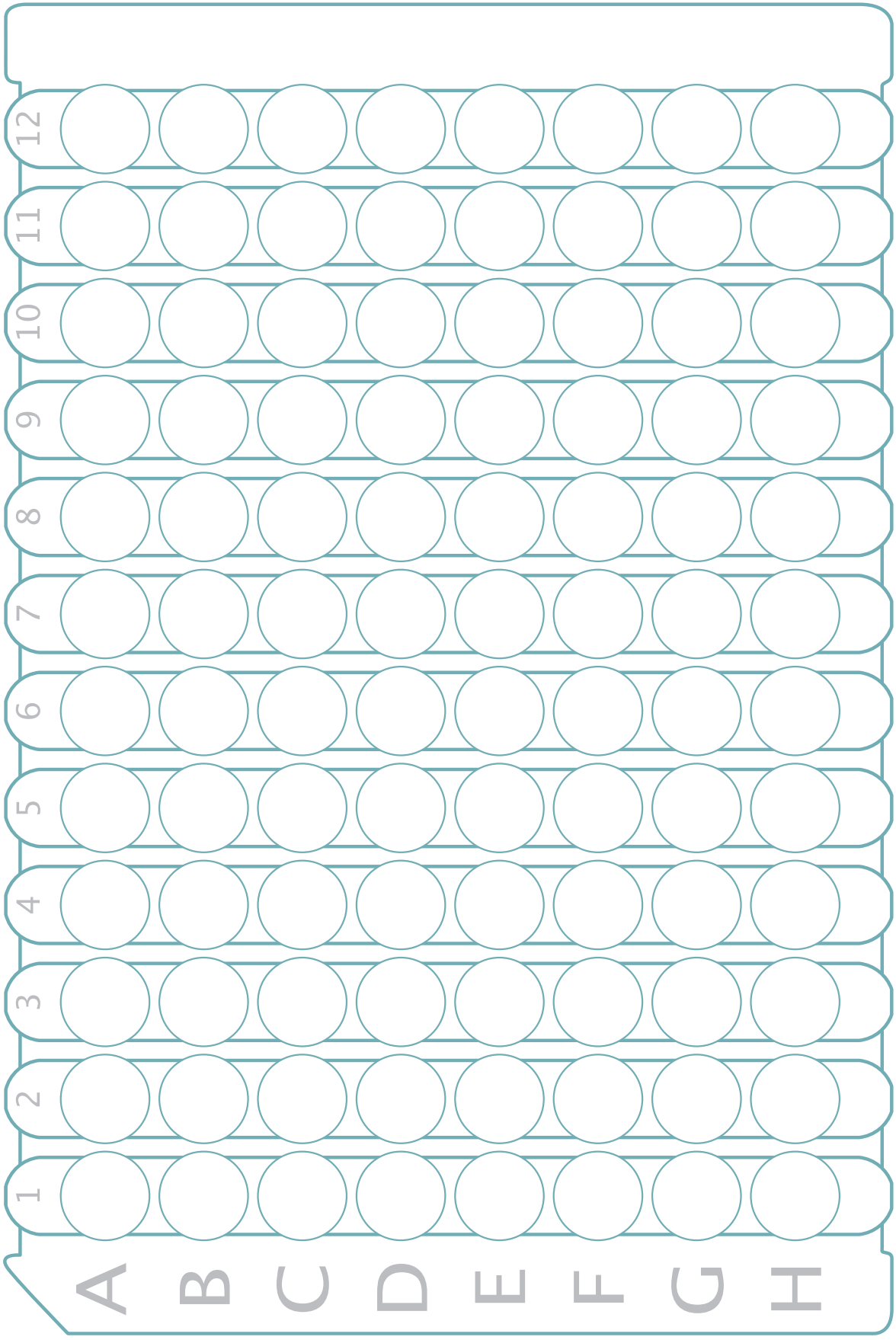
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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