Parameter™

Estradiol Assay

Catalog Number KGE014

For the quantitative determination of Estradiol 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

Estradiol, also called 17β-estradiol, estrogen, or E2, is the predominant female sex hormone. It is synthesized mainly in the ovaries and placenta, but also in some other tissues such as breast, brain, adipose, and the arterial wall (1-3). Circulating estradiol concentrations are higher in pre-menopausal women than in post-menopausal women or in men, and greatly increase during pregnancy (3, 4). Tissue concentrations of estradiol can greatly exceed circulating concentrations (3). Other natural estrogen forms include estrone (E1) and estriol (E3), while plant forms (phytoestrogens) and synthetic estrogenic compounds (xenoestrogens) also exist (2, 3). These forms may have similar or opposing effects as compared to estradiol. Estrogens mainly circulate bound to SHBG (sex hormone binding globulin), which can limit the availability of estradiol. Therefore, elimination of protein-bound estradiol, aids in the consistent measurement of bioavailable estradiol (4, 5). As a hydrophobic molecule, estradiol is thought to diffuse freely through the cell membrane when not bound to protein (3).

Widespread expression of three estrogen receptors has been described (2, 3, 6). ERα and ERβ are nuclear receptors that enhance transcription of genes containing the appropriate responsive elements when estradiol is bound. While ERα is responsible for most of the nuclear effects of estradiol, ERβ may have similar, differing, or even opposing effects on individual cell types (3, 6, 7). There is also evidence for cell membrane-tethered ERα and ERβ that participate in immediate (non-genomic) responses. GPER (G-protein-coupled estrogen receptor), also called GPR30, is an additional cell membrane receptor for estradiol that activates adenylyl cyclase signaling and transactivates the EGF receptor (3, 8). The membrane estrogen receptors mediate immediate responses to estradiol.

In the female fertility cycle, estradiol is produced by maturing follicles before ovulation and the corpus luteum after ovulation (9, 10). In controlled ovarian hyperstimulation that is performed to harvest oocytes for *in vitro* fertilization, high serum estradiol levels are associated with increased numbers of mature follicles, and subsequently with increased viability and quality of embryos (11). Estradiol stimulates endometrial proliferation to prepare for receiving a fertilized egg, and also supports implantation and placentation (2, 7). It affects pituitary gonadotrophin, leptin, and placental hormone production (2, 7, 10). Estradiol is increasingly produced by the human placenta over the term of pregnancy (2).

Estradiol is protective against cardiovascular disease in pre-menopausal females (3, 6, 12). Depletion of estrogen during menopause is associated with low-grade systemic inflammation, and post-menopausal estrogen replacement appears to protect the cardiovascular system only if there has been no gap in which a hypoestrogenic state occurs (1, 6, 13). Estradiol contributes to a robust immune system, but also contributes to increased rates of autoimmunity in females (14, 15). It regulates nitric oxide synthesis and attenuates macrophage activation and inflammatory cytokine production, while enhancing anti-inflammatory cytokine production (1, 12, 14, 15). It promotes subcutaneous fat deposition, but inhibits visceral fat deposition (16). ERα expression is required for mammary development and lactation, and high serum estradiol is associated with increased risk of post-menopausal breast cancer (3, 7, 17). It is neuroprotective (1, 2). Estradiol helps maintain pre-menopausal bone density, and serum estradiol is also positively associated with volumetric bone mass in males (3, 5, 6, 12, 18). In males, estradiol is produced in the testes by conversion from androgens, and its activity on ERα, ERβ and GPER are important in spermatogenesis (3, 19).

The Parameter Estradiol assay is a 3.5 hour competitive enzyme immunoassay designed to measure Estradiol in cell culture supernates, serum, and plasma.

PRINCIPLE OF THE ASSAY

This assay is based on the competitive binding technique. A monoclonal antibody specific for Estradiol becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess monoclonal antibody, Estradiol present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled Estradiol for sites on the monoclonal antibody. This is followed by another 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 Estradiol 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, dilute the pretreated samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, incubation time or temperature, and kit age can cause assay variability.
- 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 this assay, 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.
- 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.

MATERIALS PROVIDED & STORAGE CONDITIONS

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

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Goat anti-mouse IgG Microplate	892575	96 well polystyrene microplate (12 strips of 8 wells) coated with a goat anti-mouse IgG antibody.	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.*
Estradiol Standard	894513	30 ng of Estradiol in buffered protein base with preservatives; lyophilized.	Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
Estradiol Conjugate	894512	6 mL of Estradiol conjugated to horseradish peroxidase with red dye and preservatives.	May be stored for up to 1 month at 2-8 °C.*
Estradiol Primary Antibody Solution	894514	11 mL of mouse monoclonal antibody to Estradiol in buffered protein base with blue dye and preservatives.	
Calibrator Diluent RD5-62	895998	21 mL of a buffered protein base with preservatives.	
Pretreatment E	895996	11 mL of 0.6 N Trichloroacetic acid.	
Pretreatment F	895997	6 mL of buffer with preservatives.	
Wash Buffer 10X Concentrate	895222	100 mL of a 10-fold concentrated solution of buffered surfactant.	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895926	11 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.
- Microcentrifuge capable of ≥ 12,000 x g.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Microcentrifuge tubes for pretreatment.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards.
- Estradiol Controls (optional; available from R&D Systems).

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.

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

Some components in this kit contain ProClin® 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. Please 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. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Media containing serum may contain detectable levels of Estradiol. A media blank should be run with media containing serum.*

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, pretreat, and assay immediately or aliquot and store samples at ≤ -20 °C. Samples may be stored untreated or pretreated. 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. Pretreat and assay immediately or aliquot and store samples at ≤ -20 °C. Samples may be stored untreated or pretreated. Avoid repeated freeze-thaw cycles.

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

SAMPLE PRETREATMENT

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

1. Add 400 µL of sample and 100 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. The supernate may be cloudy until the addition of Pretreatment F.
5. Add 75 µL of Pretreatment F to 250 µL of the retained supernate. Mix well.
6. The concentration read off the standard curve must be multiplied by the dilution factor, 1.6.

REAGENT PREPARATION

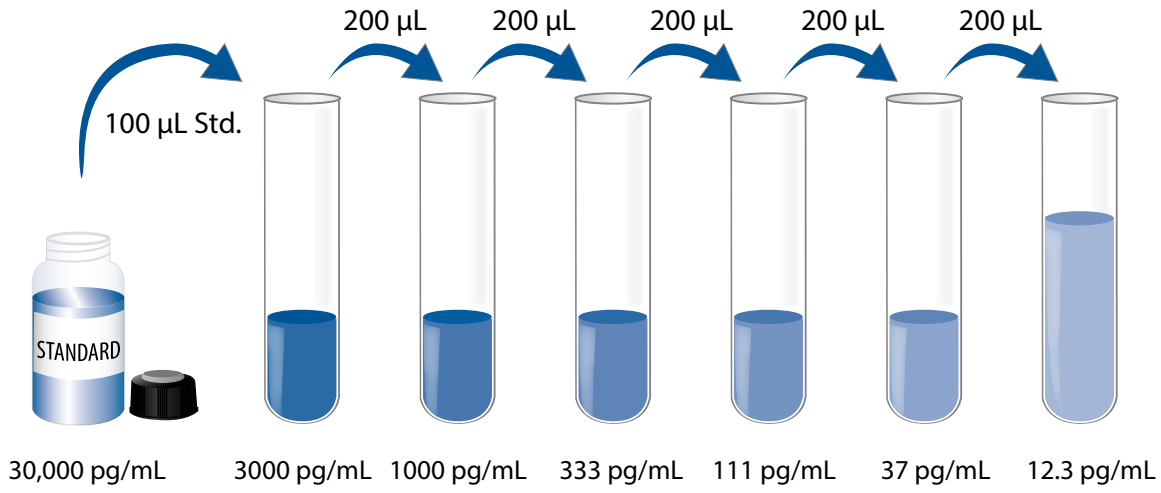
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 50 mL of Wash Buffer Concentrate into 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.

Estradiol Standard - Reconstitute the Estradiol Standard with 1.0 mL deionized or distilled water. This reconstitution produces a stock solution of 30,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle mixing prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5-62 into the 3000 pg/mL tube. Pipette 400 µL into the remaining tubes. Use the stock solution to produce a 3-fold dilution series (below). Mix each tube gently but thoroughly before the next transfer. The 3000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-62 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 Estradiol Primary Antibody Solution to each well **(excluding the NSB wells)**. 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.
4. 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.
5. Add 100 µL of Standard, control, or sample* to the appropriate wells. A plate layout is provided to record standards and samples assayed.
6. Add 100 µL of Calibrator Diluent RD5-62 to the zero standard (B_0) wells and NSB wells.
7. Add 50 µL of the Estradiol Conjugate to all wells. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on the shaker.
8. Repeat the aspiration/wash as in step 4.
9. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
10. Add 100 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.
11. 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.

*Serum and plasma samples require pretreatment. See Sample Pretreatment section.

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.

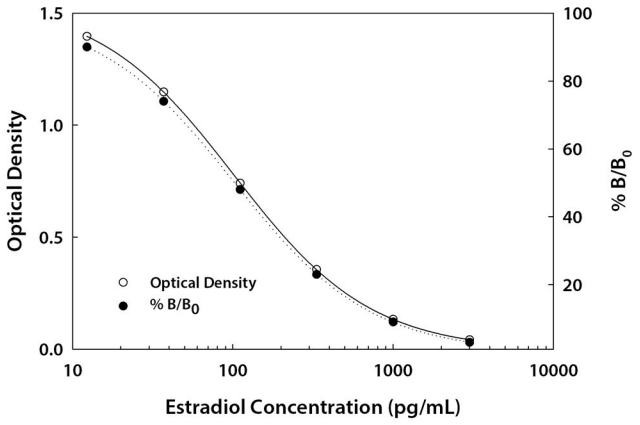
If desired, % B/B_0 can be calculated by dividing the corrected O.D. for each standard or sample by the corrected B_0 O.D. and multiplying by 100.

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

Since serum and plasma samples have been pretreated, the concentration read from the standard curve must be multiplied by the dilution factor, 1.6.

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	% B/B_0
NSB	0.008 0.009	0.009	——	——
0 (B_0)	1.551 1.556	1.554	1.545	——
12.3	1.386 1.424	1.405	1.396	90
37	1.140 1.173	1.157	1.148	74
111	0.748 0.751	0.750	0.741	48
333	0.361 0.366	0.364	0.355	23
1000	0.141 0.143	0.142	0.133	9
3000	0.050 0.052	0.051	0.042	3

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 kit components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	153	744	1419	164	765	1533
Standard deviation	13.6	26.8	76.6	9.54	61.5	116
CV (%)	8.9	3.6	5.4	5.8	8.0	7.6

RECOVERY

The recovery of Estradiol spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range %
Cell culture media (n=4)	94	81-102
Human serum* (n=4)	96	87-108
Human EDTA plasma* (n=4)	103	90-113
Human heparin plasma* (n=4)	102	88-117
Mouse EDTA plasma* (n=1)	106	98-116
Rat serum* (n=3)	116	105-124%

*Samples were pretreated and then spiked. See the Sample Pretreatment section.

SENSITIVITY

Twenty-Six assays were evaluated and the minimum detectable dose (MDD) of Estradiol ranged from 2.14-12.1 pg/mL. The mean MDD was 4.84 pg/mL.

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

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Estradiol were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Human			Mouse EDTA plasma* (n=1)	Rat serum* (n=3)
			Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)		
1:2	Average % of Expected	100	103	101	106	104	112
	Range (%)	88-115	96-109	93-107	92-119	———	101-119
1:4	Average % of Expected	102	98	102	98	90	113
	Range (%)	95-111	92-104	89-113	85-106	———	98-122
1:8	Average % of Expected	101	101	98	98	101	113
	Range (%)	88-111	94-106	93-105	86-105	———	102-121
1:16	Average % of Expected	104	105	103	101	103	117
	Range (%)	93-122	96-113	100-104	92-107	———	109-124

*Samples were pretreated and then spiked. See the Sample Pretreatment section.

SAMPLE VALUES

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

Male Samples	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Male serum (n=18)	48.8	94	ND-78.8
Male EDTA plasma (n=18)	45.8	100	21.9-87.9
Male heparin plasma (n=18)	40.4	94	ND-71.3

Female Samples	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Female serum (n=17)	64.8	100	26.4-131
Female EDTA plasma (n=17)	60.3	100	21.3-132
Female heparin plasma (n=17)	56.1	94	ND-129

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Mouse serum (n=7)	59.5	100	45.5-75.0
Rat serum (n=3)	81.3	100	71.7-91.5

Cell Culture Supernates - Human peripheral blood lymphocytes (PBLs) were cultured in DMEM supplemented with 5% fetal calf serum, 50 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 for 1 and 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of Estradiol. All samples had non-detectable levels of Estradiol.

SPECIFICITY

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

Substances:

5α-Androstan-17β-ol-3-one
6β-Hydroxytestosterone
11α-Hydroxytestosterone
β-Estradiol 3-(β-D-glucuronide)
β-Estradiol 3-sulfate
Androsterone
Aldosterone
Cortisol
Cortisone
Deoxycorticosterone
Epitestosterone
Estrone 3-sulfatee
Prednisolone
Prednisone
Testosterone
trans-Dehydroantrosterone

Some cross-reactivity was observed with the following:

Compound	% Cross-reactivity
17α-ethynylestradiol	0.07
17β-estradiol 3-Benzoate	0.03
Estriol	0.86
Estrone	0.26
Progesterone	0.06

Sex Hormone Binding Globulin (SHBG) interferes in this assay. Sample pretreatment removes SHBG and estradiol-bound SHBG.

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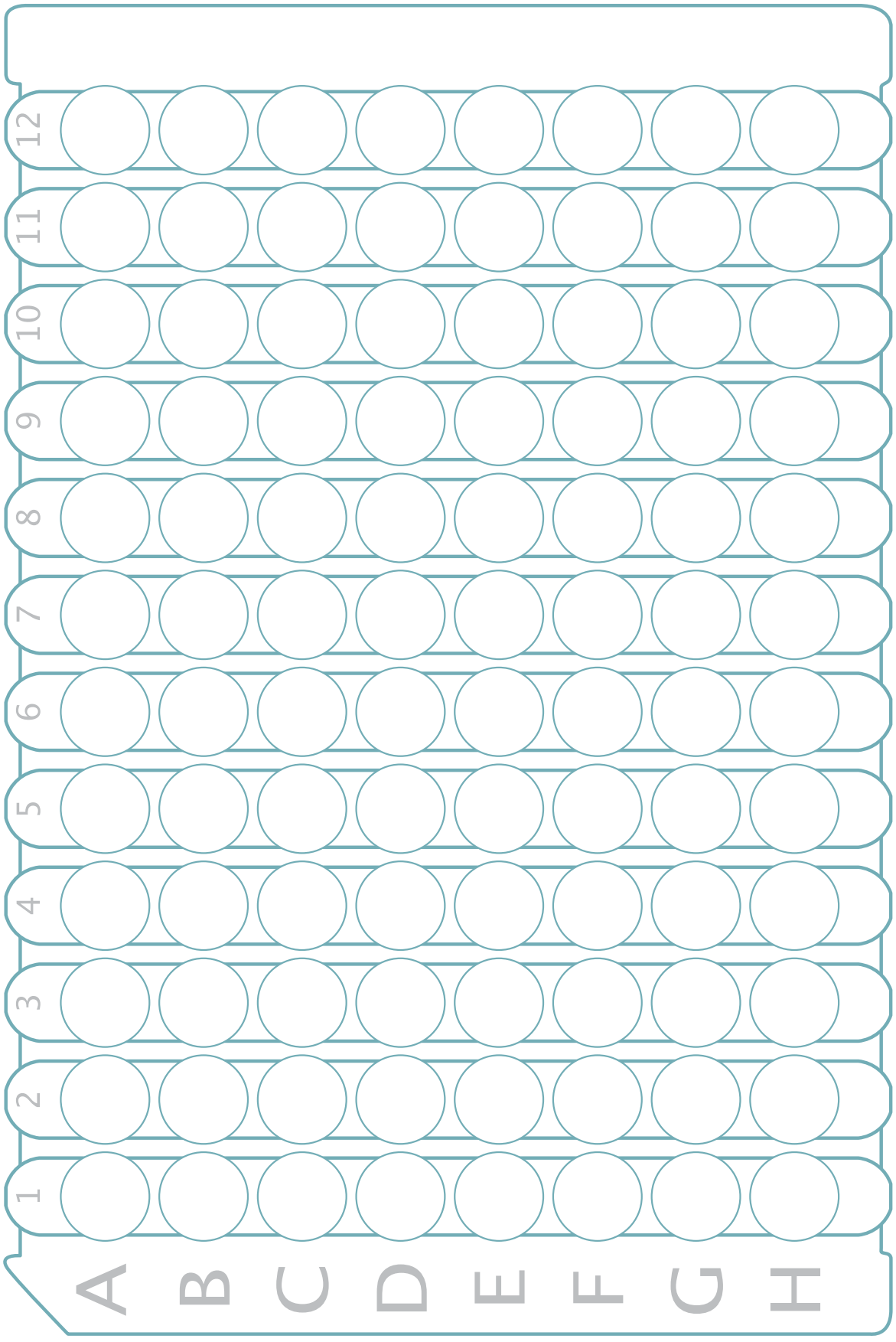
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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