Parameter™

Testosterone Assay

Catalog Number KGE010
SKGE010
PKGE010

For the quantitative determination of Testosterone 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

Testosterone is a sex steroid hormone primarily produced by the testicular Leydig cells (1). Two metabolites of this androgen are dihydroTestosterone (DHT) and estradiol. Testosterone is traditionally identified with spermatogenesis in the seminiferous tubules and development and maintenance of primary and secondary sex characteristics (2, 3). It is also implicated in immunosuppression (4-8), bone density (9-11), vasodilation (12-16), prostate cancer (17-20), diabetes (21), and synaptic plasticity (22).

As with other steroid hormones, Testosterone production is regulated by pituitary peptide hormones. Upon stimulation by gonadatropin releasing hormone (GnRH), luteinizing hormone (LH) is released from the anterior pituitary and interacts with membrane receptors on the Leydig cells to induce Testosterone synthesis via cAMP-dependent cholesterol metabolism (2, 3, 23). Follicle stimulating hormone (FSH), also released in response to GnRH, may indirectly influence Leydig cell function and steroidogenesis via its effect on Sertoli cells (24). Among numerous factors, GnRH release is influenced by Testosterone levels in a classic negative feedback fashion. In adult male humans, approximately 5 mg of Testosterone is synthesized daily and released in a diurnal pattern with serum and salivary levels peaking in the morning (25, 26). Most circulating Testosterone is bound to proteins, such as sex hormone binding globulin (SHBG) or albumin. Free Testosterone and albumin-bound Testosterone are considered bioactive (27).

Free Testosterone diffuses across the cell membrane and interacts with cytosolic androgen receptor (AR) (28). The hormone-receptor complex translocates to the nucleus and alters DNA transcription (29). AR activation has also been associated with non-genomic effects that appear critical to Testosterone action, such as protein kinase activation and intracellular calcium flux (30-32). In the presence of the enzyme 5α-reductase, Testosterone is converted to DHT, an AR ligand with five times the binding affinity relative to Testosterone. Several co-repressors and co-activators may alter the effect of Testosterone on the target cell, explaining tissue-specific responses (33, 34). Testosterone can also affect target tissue by its conversion to estradiol via aromatase and interaction with an estrogen receptor.

The Parameter™ Testosterone assay is a 4.5 hour competitive enzyme immunoassay designed to measure Testosterone 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 Testosterone becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess monoclonal antibody, Testosterone present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled Testosterone 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 Testosterone 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. Until all factors have been tested in this assay, the possibility of interference cannot be excluded.
- **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 # KGE010	CATALOG # SKGE010	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Goat anti-mouse IgG Microplate	892575	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a goat anti-mouse IgG 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.*
Testosterone Conjugate	893263	1 vial	6 vials	6 mL/vial of Testosterone conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Testosterone Standard	893265	1 vial	6 vials	Testosterone in buffer with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Primary Antibody Solution	893264	1 vial	6 vials	6 mL/vial of a mouse monoclonal antibody for Testosterone in buffer with blue dye and preservatives.	
Calibrator Diluent RD5-48	895911	2 vials	12 vials	21 mL/vial of a buffered protein base 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	Adhesive strips.	

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

KGE010 contains sufficient materials to run an ELISA on one 96 well plate.
SKGE010 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PKGE010). 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.

PRECAUTIONS

Care should be taken when handling the standard because of the known and unknown effects of testosterone.

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.

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.
- Test tubes for dilution of standards and samples.
- Testosterone Controls (optional; R&D Systems®, Catalog # QC165).

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.

Note: *Media containing serum may contain detectable levels of Testosterone. 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 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.*

SAMPLE PREPARATION

Cell culture supernate, serum, and plasma samples require a 10-fold dilution. A suggested 10-fold dilution is 50 µL of sample + 450 µL of Calibrator Diluent RD5-48.

REAGENT PREPARATION

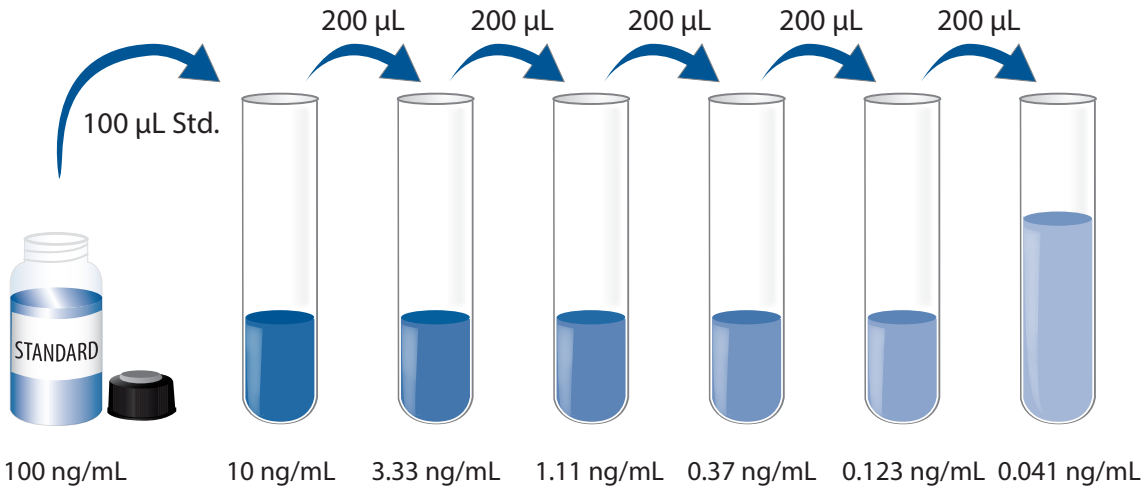
Bring all reagents to room temperature before use.

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 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.

Testosterone Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Testosterone 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 RD5-48 into the 10 ng/mL tube. Pipette 400 µL of calibrator diluent 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 RD5-48 serves as the zero standard (B_0) (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.

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 50 µL of Primary Antibody Solution to each well **excluding the non-specific binding (NSB) wells.** All wells except the NSB wells will now be blue in color.
4. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
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 100 µL of Calibrator Diluent RD5-48 to the NSB wells.
7. Add 100 µL of Calibrator Diluent RD5-48 to the zero standard (B_0) wells.
8. Add 100 µL of standard, control, or sample* to the remaining wells. A plate layout is provided to record standards and samples assayed.
9. Add 50 µL of the Testosterone Conjugate to each well. Cover with the adhesive strip provided.
10. Incubate for 3 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
11. Repeat the wash as in step 5.
12. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
13. 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.
14. 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 require dilution. See Sample Preparation 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 parameter 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 Testosterone corresponding to the mean absorbance from the standard curve.

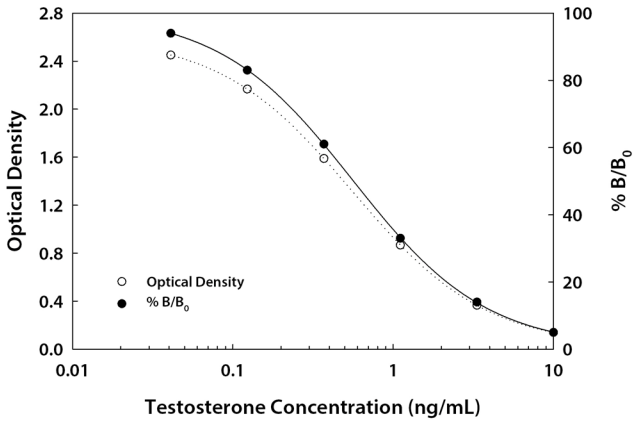
Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

To convert results from ng/mL to nmol/L, use the following equation:

1.0 ng/mL = 3.47 nmol/L

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	% B/B_0
NSB	0.021 0.027	0.024	—	—
0 (B_0)	2.604 2.645	2.625	2.601	—
0.041	2.465 2.483	2.474	2.450	94
0.123	2.175 2.205	2.190	2.166	83
0.37	1.577 1.647	1.612	1.588	61
1.11	0.880 0.902	0.891	0.867	33
3.33	0.383 0.395	0.389	0.365	14
10	0.159 0.164	0.162	0.138	5

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)	1.25	3.50	5.80	1.26	3.67	6.03
Standard deviation	0.05	0.11	0.17	0.07	0.23	0.41
CV (%)	4.0	3.1	2.9	5.6	6.3	6.8

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media* (n=4)	103	95-117%
Serum* (n=4)	93	86-104%
Heparin plasma* (n=4)	92	84-101%
EDTA plasma* (n=4)	94	85-107%

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

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Testosterone 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	98	96	96	101
	Range (%)	95-101	85-109	86-106	87-112
1:4	Average % of Expected	97	97	100	103
	Range (%)	93-100	86-115	88-110	96-111
1:8	Average % of Expected	94	101	104	106
	Range (%)	91-96	98-105	99-107	101-115

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

SENSITIVITY

Thirty-three assays were evaluated and the minimum detectable dose (MDD) of Testosterone ranged from 0.012-0.041 ng/mL. The mean MDD was 0.030 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 - Samples from apparently healthy volunteers were evaluated for the presence of Testosterone in this assay. No medical histories were available for the donors used in this study.

Male Samples	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=14)	7.04	4.19-11.8	2.13
EDTA plasma (n=14)	6.55	3.62-11.7	2.07
Heparin plasma (n=14)	5.84	2.85-8.58	1.66

Female Samples	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=20)	1.56	0.66-2.36	0.50
EDTA plasma (n=20)	1.30	0.50-2.19	0.44
Heparin plasma (n=20)	1.24	0.51-1.92	0.38

Cell Culture Supernates - Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 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 supernate were removed on days 1 and 5 and assayed for levels of Testosterone. 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 Testosterone control were assayed for interference. Cross-reactivity is listed below.

Condition	% Cross-reactivity
11α-hydroxytestosterone	23%
6β-hydroxytestosterone	20%
5α-androstan-17β-ol-3-one	2.6%
Epitestosterone	0.27%
Androsterone	< 0.1%
Estradiol	< 0.1%
Human SHBG*	< 0.1%
Prednisolone	< 0.1%
Prednisone	< 0.1%
Progesterone	< 0.1%
Trans-dehydroandrosterone	< 0.1%

*Interferes at levels ≥ 100 ng/mL in this assay.

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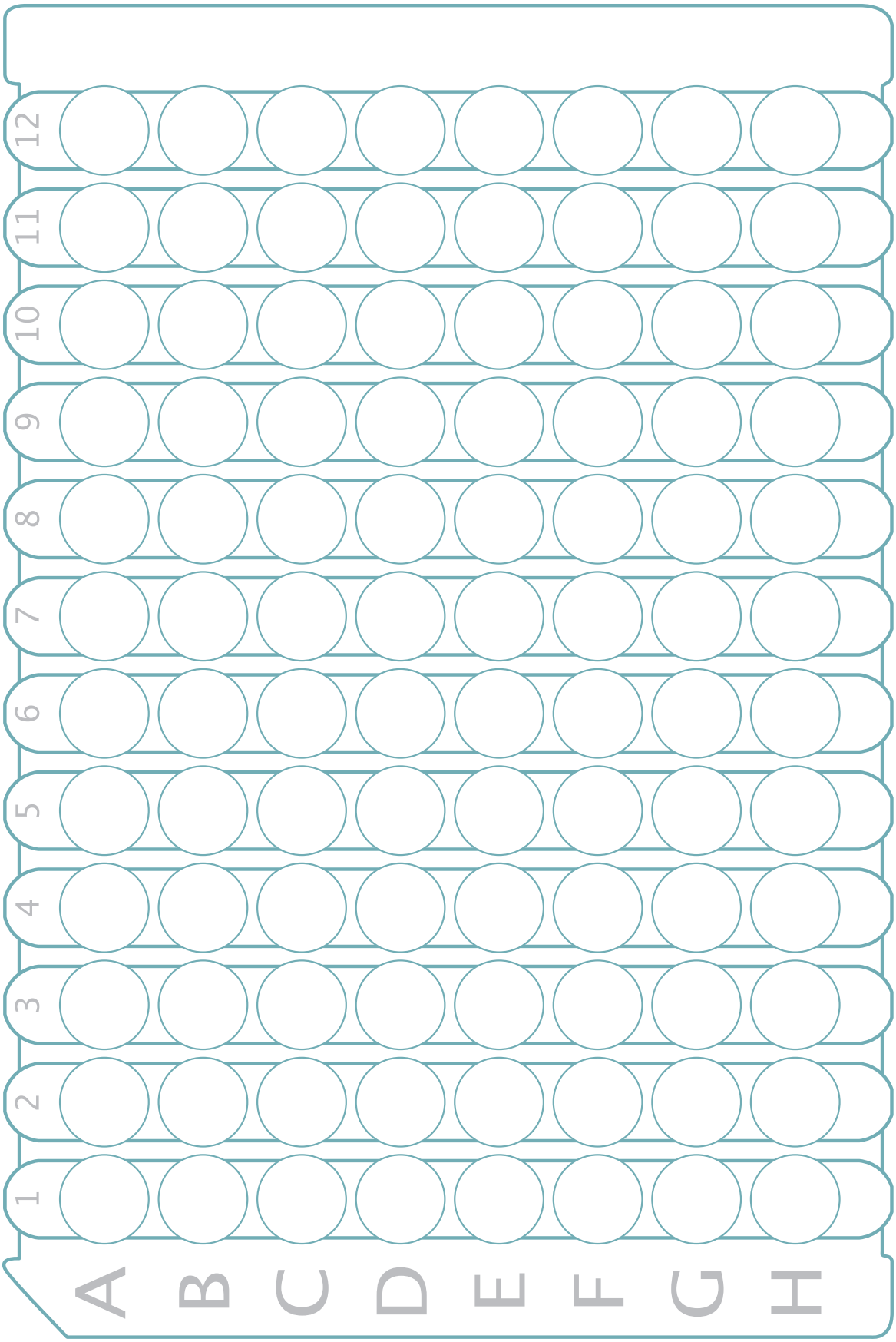
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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