Quantikine® ELISA

Rat/Mouse Cytochrome c Immunoassay

Catalog Number MCTC0

For the quantitative determination of rat or mouse Cytochrome c concentrations in cell lysates and subcellular fractions.*

**Optimal fractionation protocols must be determined by the end-user.*

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

Rat/mouse Cytochrome c is a 104 amino acid polypeptide (1) that participates in oxidative phosphorylation (2) and apoptosis (3). The protein is synthesized in the cytosol as a 12 kDa apoprotein. The apoprotein is transported across the outer mitochondrial membrane into the intermitochondrial membrane space where a heme is covalently attached to the N-terminus by Cytochrome c heme lyase (4). Cytochrome c undergoes a conformational change and is normally sequestered in the intermitochrondrial membrane space. Rat and mouse Cytochrome c have 100% amino acid identity (1) and rat/mouse Cytochrome c has 91% amino acid identity with human Cytochrome c (5). Although the somatic form of Cytochrome c is clearly the most widely expressed form, a testes-specific form of Cytochrome c also exists (6). Rat testicular and somatic forms differ by 15 amino acids. Rat testes Cytochrome c and mouse testes Cytochrome c differ by four amino acids.

Cytochrome c is actively involved in the oxidative phosphorylation pathway. Cytochrome c transports electrons from the Cytochrome c reductase complex to the Cytochrome c oxidase complex (2). Transport of electrons along the chain of electron donors and acceptors is required for the generation of ATP during respiration. In response to many apoptotic signals, Cytochrome c is released from the mitochondria into the cytosol (7-10). When released from the mitochondria Cytochrome c activates an apoptotic program via caspase cascades (7, 9, 11-13). Growth factor withdrawal is one event that triggers apoptotic signals. Some apoptotic signals appear to involve relocation of cytosolic Bax (14-16) and/or Bad (17) to the outer mitochondrial membrane. Relocation or conformational changes of Bax/Bad or other pro-apoptotic Bcl-2 family members (18) induces the release of Cytochrome c into the cytosol. Released Cytochrome c then forms a complex with Apaf-1 (apoptotic protease activating factor-1), caspase-9, and dATP (7, 19, 20). Formation of this complex activates caspase-9, which then activates downstream caspases (11-13, 19). Activated caspases then disassemble the cell (21).

The Quantikine Rat/Mouse Cytochrome c Immunoassay is a 2.5 hour solid phase ELISA designed to measure rat and mouse Cytochrome c. It contains natural rat Cytochrome c and antibodies raised against Cytochrome c, and because of the 100% sequence identity of rat and mouse Cytochrome c, this kit can be used to accurately quantitate natural rat or mouse Cytochrome c in cell lysates and subcellular fractions.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique and is designed to replace Western blot. A monoclonal antibody specific for rat/mouse Cytochrome c has been pre-coated onto a microplate. Conjugate, Standards, Control, and samples are pipetted into the wells and any rat/mouse Cytochrome c present is bound by the immobilized antibody and the enzyme-linked monoclonal antibody specific for rat/mouse Cytochrome c. Following a wash to remove any unbound substances, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of rat/mouse Cytochrome c bound in the initial step. The sample values are then read off the standard curve.

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.
- This kit is validated for cell lysates and subcellular fractions only. Serum samples cannot be assayed in this kit.
- This assay is sensitive to high concentrations of DTT; if present, dilute the sample with Calibrator Diluent RD5-18 to a final concentration of ≤ 0.1 mM.
- If samples generate values higher than the highest standard, dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard 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.
- 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.

PRECAUTIONS

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.

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
Rat/Mouse Cytochrome c Microplate	890020	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for rat/mouse Cytochrome c.	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.*
Rat/Mouse Cytochrome c Standard	890031	200 ng of natural rat Cytochrome c in a 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.
Rat/Mouse Cytochrome c Control	890059	1 vial of natural rat Cytochrome c in a buffered protein base with preservatives; lyophilized. The concentration range of rat/mouse Cytochrome c after reconstitution is shown on the vial label. The assayed value of the Control should be within the range specified on the label.	
Rat/Mouse Cytochrome c Conjugate	890030	12 mL of a monoclonal antibody specific for rat/mouse Cytochrome c conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Calibrator Diluent RD5-18	895335	2 vials (21 mL/vial) of a buffered surfactant with preservatives.	
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	895174	23 mL of diluted hydrochloric 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.
- Triton™ X-100 for sample preparation.
- Centrifuge capable of obtaining 16,000 x g for sample preparation.
- **Polypropylene** test tubes for dilution of standards.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

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

Note: *Serum samples are not suitable for use in this assay.*

SAMPLE PREPARATION

The final concentration of dithiothreitol or other sulfhydryl reducing agents in the sample should be ≤ 0.1 mM.

Quantitation of total Cytochrome c in cultured cells:
Before measuring the amount of Cytochrome c in subcellular compartments, the total amount of Cytochrome c in the cells should be determined. When the amount of total Cytochrome c in the cells is known, the proper dilutions of subcellular fractions* required to obtain optical density readings on the linear portion of the standard curve can be calculated. To determine the total amount of Cytochrome c in the cells:

1. Wash cells three times with PBS to remove serum components.
2. Solubilize 1 x 10^6 cells/mL in PBS containing 0.5% Triton X-100 for 10 minutes at 2-8 °C.
3. Centrifuge the extract at 16,000 x g for 10 minutes.
4. Collect the supernate and serially dilute an aliquot with Calibrator Diluent RD5-18 to 3-fold, 10-fold, and 100-fold.
5. After the total Cytochrome c has been determined, dilutions of the subcellular fractions* in Calibrator Diluent RD5-18 that have optical density readings within the standard curve range can be calculated.

As a reference point for determining the total amount of Cytochrome c in cells, cells typically contained 19-63 ng of Cytochrome c per 1 x 10^6 cells. Amounts may vary in different cell types.

Quantitation of Cytochrome c in subcellular fractions* obtained from cultured cells:
For information on using subcellular fractionation to determine the distribution of Cytochrome c in apoptotic cells, see references 10, 22-24. After the total Cytochrome c has been determined, dilute the appropriate subcellular fractions in Calibrator Diluent RD5-18 to have an optical density reading within the standard curve range.

**A procedure for subcellular fractionation has not been validated for this assay.*

Quantitation of Cytochrome c in isolated mitochrondria and released from isolated mitochondria:
When quantitating Cytochrome c in experiments examining the *in vitro* release of Cytochrome c from enriched mitochondria, determine the total amount of Cytochrome c in the mitochondrial fraction before measuring the amount released from or retained in the mitochondria.

1. Solubilize an aliquot of mitochondria preparation in PBS containing 0.5% Triton X-100 for 10 minutes at 2-8 °C .
2. Centrifuge at 16,000 x g for 10 minutes.
3. Serially dilute the supernate with Calibrator Diluent RD5-18 to protein concentrations between 1-20 µg/mL mitochondrial protein.
4. After total Cytochrome c in the mitochondria preparation has been determined, the amount and dilutions of samples in Calibrator Diluent RD5-18 required to obtain results in the standard curve range can be calculated. Follow the Assay Procedure (page 7) to determine Cytochrome c in the diluted samples.

In vitro assays for release of Cytochrome c from intact mouse liver mitochondria typically contained 250-300 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.01 mM dithiothreitol, 10 mM KH_2PO_4, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin, and 1 µM Boc-Asp-fmk (Enzyme Systems). The pH was adjusted to 7.5 with KOH. For more information on the release of Cytochrome c from isolated mouse liver, rat liver, or cultured cells, see references 10, 19, 25-28.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

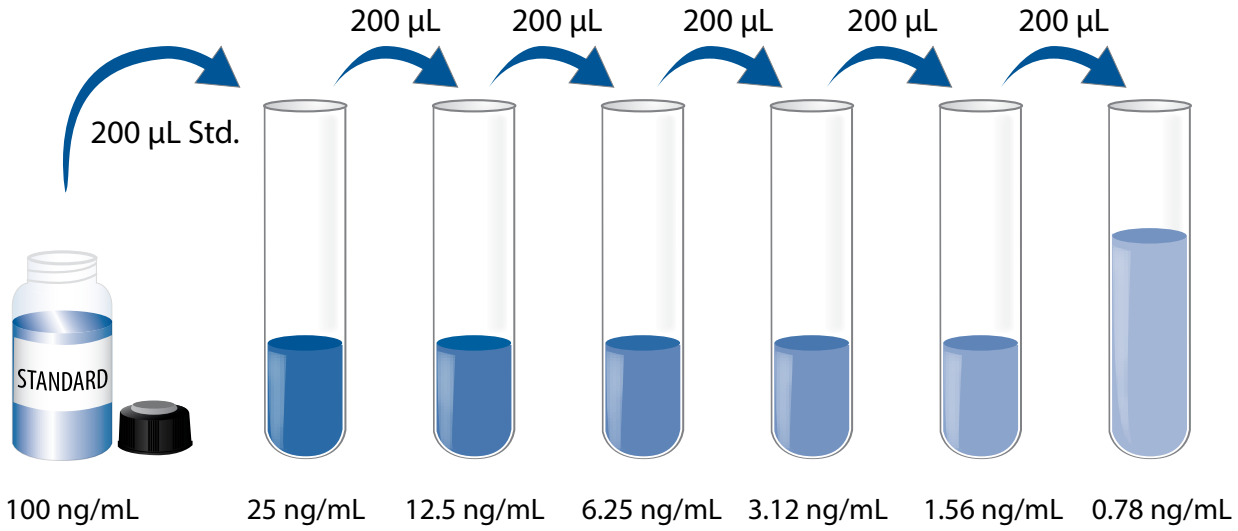
Rat/Mouse Cytochrome c Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the Control undiluted.

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. 100 µL of the resultant mixture is required per well.

Rat/Mouse Cytochrome c Standard - Reconstitute the Rat/Mouse Cytochrome c Standard with 2.0 mL of Calibrator Diluent RD5-18. Do not substitute other diluents. This reconstitution produces a stock solution of 100 ng/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 600 µL of Calibrator Diluent RD5-18 and 200 µL of the stock solution (100 ng/mL) into the 25 ng/mL tube and mix thoroughly. Pipette 200 µL of Calibrator Diluent RD5-18 into the remaining tubes. Use the 25 ng/mL standard to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The 25 ng/mL standard serves as the high standard. Calibrator Diluent RD5-18 serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

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

1. Prepare all reagents, working standards, control, 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 75 µL of Rat/Mouse Cytochrome c Conjugate to each well.
4. Add 50 µL of Standard, Control, or sample per well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided for a record of samples and standards assayed.
5. Aspirate each well and wash, repeating the process four times for a total of five 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 Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
7. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
8. 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

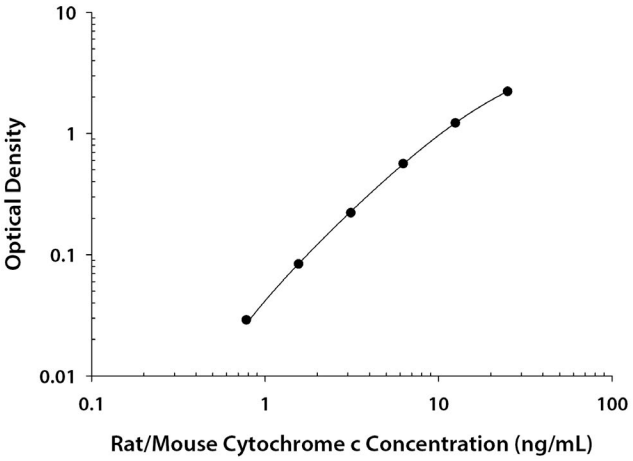
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) 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 the graph. The data may be linearized by plotting the log of the rat/mouse Cytochrome c concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If 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.



(ng/mL)	O.D.	Average	Corrected
0	0.035 0.037	0.036	—
0.78	0.064 0.066	0.065	0.029
1.56	0.123 0.116	0.120	0.084
3.12	0.260 0.257	0.258	0.222
6.25	0.604 0.595	0.600	0.564
12.5	1.269 1.242	1.256	1.220
25	2.293 2.227	2.260	2.224

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	1.61	8.82	13.4	1.72	8.91	13.5
Standard deviation	0.02	0.09	0.19	0.06	0.31	0.59
CV (%)	1.24	1.02	1.42	3.49	3.48	4.37

RECOVERY

The recovery of rat/mouse Cytochrome c spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
PBS/0.5% Triton X-100	100%	96-106%
PBS/1% Triton X-100	98%	95-101%
PBS/0.05% Tween™ 20	101%	100-102%
PBS/0.01% Saponin	94%	90-98%

LINEARITY

To assess the linearity of the assay, five or more samples spiked with various concentrations of rat/mouse Cytochrome c in each matrix were diluted with Calibrator Diluent and then assayed.

Sample	Dilution	Observed (ng/mL)	Expected (ng/mL)	$\frac{\text{Observed}}{\text{Expected}} \times 100$
PBS/0.01% Tween 20	Spiked	22.0	———	———
	1:2	11.0	11.0	100%
	1:4	5.6	5.5	102%
	1:8	2.8	2.8	100%
	1:16	1.5	1.4	107%
PBS/0.5% Triton X-100	Spiked	21.0	———	———
	1:2	10.0	10.0	100%
	1:4	5.4	5.0	108%
	1:8	2.7	2.5	108%
	1:16	1.4	1.2	117%

SENSITIVITY

The minimum detectable dose (MDD) of rat/mouse Cytochrome c is typically less than 0.5 ng/mL.

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

CALIBRATION

This assay is calibrated against a highly purified natural rat Cytochrome c protein isolated from rat heart. Based upon amino acid analysis, the molecular weight of this protein is 12.1 kDa, which contains less than 5% of the reduced form of Cytochrome c. This immunoassay recognizes both the oxidized and reduced forms of Cytochrome c.

SAMPLE VALUES

CTLL-2 mouse cytotoxic T cells (1 x 10^6 cells/mL) were cultured for 5-7 days in RPMI and supplemented with 10% FBS. The cells (0.5 x 10^6) were washed 3 times with PBS and lysed with 0.5% Triton X-100. The preparation was then centrifuged for 10 mintues at 1000 x g. An aliquot of the cell culture supernate was removed, assayed for Cytochrome c, and measured 190 ng/mL.

DA-1a mouse myeloid leukemia cells (1 x 10^6 cells/mL) were cultured for 5 days in RPMI supplemented with 8% FCS, 2 mM glutamine and 10 ng/mL rmIL-3. The cells (0.5 x 10^6) were washed 3 times with PBS and lysed with 0.5% Triton X-100. The preparation was then centrifuged for 10 minutes at 1000 x g. An aliquot of the cell culture supernate was removed, assayed for Cytochrome c, and measured 633 ng/mL.

Mouse liver mitochondria (enriched) supernate was assayed for mouse Cytochrome c and measured 1.5 ng of Cytochrome c per µg protein.

SPECIFICITY

This assay recognizes the reduced and oxidixed forms of natural rat and mouse Cytochrome c.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5-18 and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range rat/mouse Cytochrome c control were assayed for interference. No significant cross-reactivity or interference was observed.

Natural proteins:

equine Cytochrome c
porcine Cytochrome c

Other factors:

apo-Cytochrome c

Natural human Cytochrome c cross-reacts approximately 7.2% in this assay.

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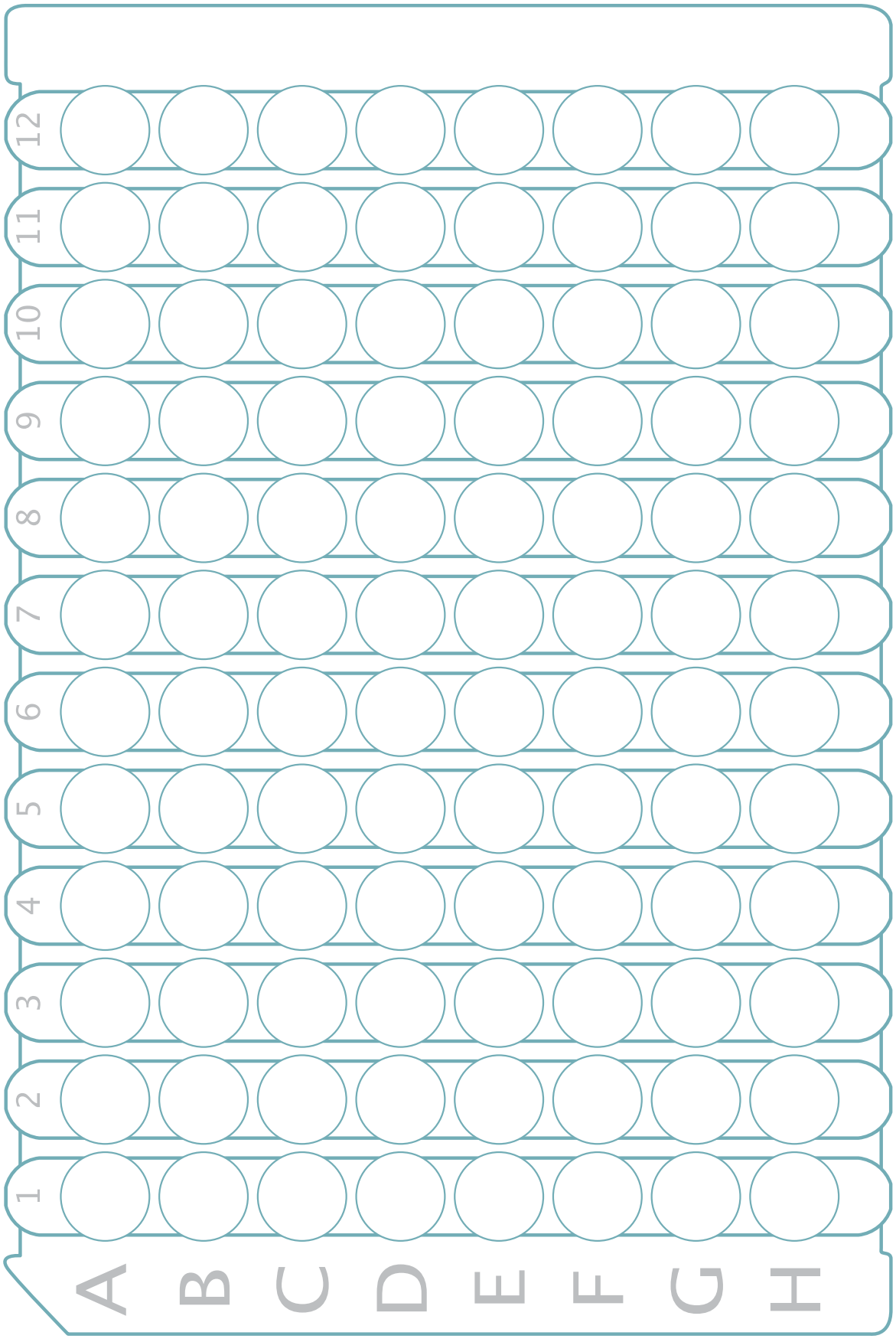
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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