Quantikine® ELISA

Human Fas/TNFRSF6 Immunoassay

Catalog Number DFS00

For the quantitative determination of human Fas 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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MANUFACTURED AND DISTRIBUTED BY:

USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

Fas, also known as APO-1, or CD95, is a cell-surface receptor that transduces apoptotic signals from Fas ligand (FasL) (1, 2). It is a glycoprotein with a mass estimated at 43 to 48 kDa (3, 4). Fas is a member of the Tumor Necrosis Factor Receptor Superfamily (TNFRSF), and it shares a cytoplasmic motif with TNF RI, referred to as the 'death domain', that binds cytoplasmic signaling molecules to trigger the cytoplasmic apoptotic signal (1, 2, 5). Fas is expressed to a large extent on activated T and B lymphocytes, and on malignant lymphoid cells. To a lesser extent, Fas is expressed on cells from liver, heart, kidney, ovaries, and on many other malignant cells.

FasL, the physiological agonist for Fas, is also a transmembrane protein (1, 2, 5) with homology to the TNF family in its extracellular domain. FasL is expressed primarily by activated T lymphocytes and by cells of the small intestine and lung. Mice with mutations in either Fas or FasL exhibit accumulation of activated lymphocytes and classical autoimmune symptoms, suggesting that a major function of Fas-mediated apoptosis is the elimination of activated immune cells from the peripheral circulation (6). Similarly, humans with autoimmune lymphoproliferative syndrome have mutations in Fas (7, 8).

Fas and FasL have been observed as soluble molecules in addition to their membrane-associated forms, suggesting additional complexity to regulation of this apoptotic mechanism (9-11). Soluble Fas (sFas) arises from alternatively spliced mRNA, leading to proteins with deletion or disruption of the single membrane-spanning domain (9, 10). Five alternatively spliced Fas mRNAs have been described (10), each protein detected in the supernate of cultures of peripheral blood mononuclear cells or certain tumor cell lines. Each sFas inhibited apoptosis induced by FasL (9, 10), and tumor-cell lines resistant to anti-Fas were shown to produce alternatively spliced Fas, thereby making them less sensitive to FasL. In addition, plasma Fas can arise by exfoliation of membrane vesicles, which also inhibit FasL-induced apoptosis (12). Serum Fas has been reported to be elevated in cancer patients (13-16), possibly originating in the tumor cell itself (17), and in autoimmune diseases (18, 19).

The Quantikine® Human Fas/TNFRSF6 Immunoassay is a 4.5 hour solid phase ELISA designed to measure human Fas in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant human Fas/Fc chimera and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant factor. Results obtained measuring natural human Fas showed dose-response 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 natural human Fas.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Fas has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Fas present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Fas 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 Fas 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, further 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.
- 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 #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Fas Microplate	890662	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Fas.	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.*
Human Fas Conjugate	890663	21 mL of a polyclonal antibody specific for human Fas conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Fas Standard	890664	Recombinant human Fas/Fc chimera in a buffered protein base with preservatives; lyophilized. *Refer to vial label for reconstitution volume.*	
Assay Diluent RD1-8	895465	11 mL of a buffered protein base with preservative.	
Calibrator Diluent RD5L	895028	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:2 in this assay.*	
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	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	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.
- 50 mL and 500 mL graduated cylinders.
- Test tubes for dilution of standards and samples.
- Human Fas Controls (optional; R&D Systems®, Catalog # QC22).

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 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 and 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, heparin, or citrate 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.

SAMPLE PREPARATION

Serum and plasma samples require at least a 10-fold dilution prior to assay. A suggested 10-fold dilution is 25 µL of sample + 225 µL of Calibrator Diluent RD5L (diluted 1:2).*

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

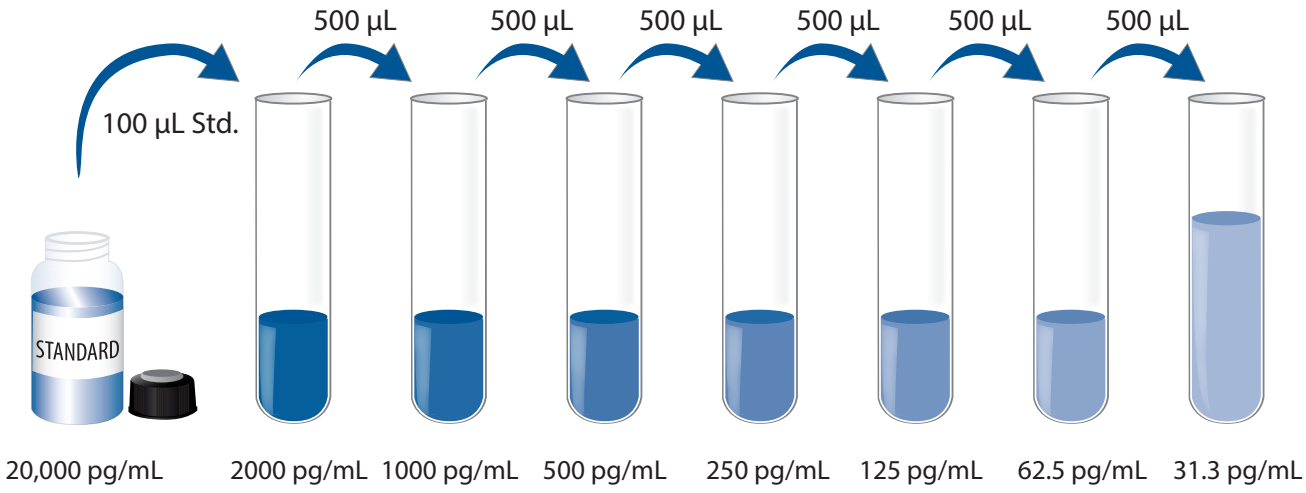
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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 RD5L (diluted 1:2) - Add 20 mL of Calibrator Diluent RD5L to 20 mL deionized or distilled water to prepare 40 mL of Calibrator Diluent RD5L (diluted 1:2).

Human Fas Standard - **Refer to vial label for reconstitution volume.** Reconstitute the Human Fas Standard with deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5L (diluted 1:2) into the 2000 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 2000 pg/mL standard serves as the high standard. Calibrator Diluent RD5L (diluted 1:2) 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, samples, and controls 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 RD1-8 to each well.
4. Add 100 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record 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 Human Fas Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
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. **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.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

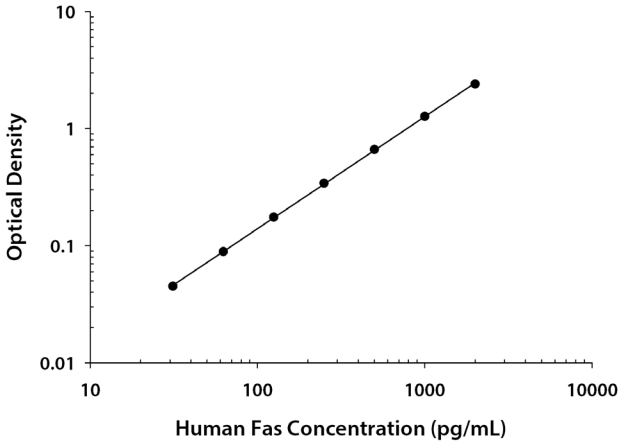
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 human Fas concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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.



(pg/mL)	O.D.	Average	Corrected
0	0.068 0.070	0.069	—
31.3	0.113 0.112	0.112	0.043
62.5	0.158 0.156	0.157	0.088
125	0.249 0.244	0.246	0.177
250	0.424 0.431	0.428	0.359
500	0.788 0.783	0.786	0.717
1000	1.456 1.440	1.448	1.379
2000	2.790 2.656	2.723	2.654

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)	130	804	1424	140	811	1391
Standard deviation	5.0	23.3	65.2	9.4	31.8	40.0
CV (%)	3.8	2.9	4.6	6.7	3.9	2.9

RECOVERY

The recovery of human Fas 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	92-107%
Serum* (n=5)	97	91-110%
Heparin plasma* (n=5)	94	88-109%
EDTA plasma* (n=5)	97	86-111%
Citrate plasma* (n=5)	98	82-116%

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

LINEARITY

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

		Cell culture media (n=5)	Serum* (n=5)	EDTA plasma* (n=5)	Heparin plasma* (n=5)	Citrate plasma* (n=5)
1:2	Average % of Expected	104	101	101	100	103
	Range (%)	102-107	99-103	95-106	97-103	100-104
1:4	Average % of Expected	105	103	101	103	105
	Range (%)	102-110	97-107	90-109	95-113	102-110
1:8	Average % of Expected	106	100	98	98	100
	Range (%)	102-113	95-108	90-109	92-103	94-107

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

SENSITIVITY

The minimum detectable dose (MDD) of human Fas is typically less than 20 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 NS0-expressed recombinant human Fas/Fc chimera produced at R&D Systems®.

SAMPLE VALUES

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

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=60)	9406	4792-17,150	2530
EDTA plasma (n=35)	8744	5941-13,460	2150
Heparin plasma (n=35)	8397	5462-11,660	1661
Citrate plasma (n=35)	7475	4260-10,530	1416

Cell Culture Supernates - Human peripheral blood mononuclear cells (5 x 10^6 cells/mL) were cultured in RPMI supplemented with 5% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed on days 1, 3, and 5 and assayed for levels of human Fas.

Sample Type	Day 1 (pg/mL)	Day 3 (pg/mL)	Day 5 (pg/mL)
Unstimulated	111	559	907
Stimulated	401	1847	2896

SPECIFICITY

This assay recognizes natural and recombinant human Fas.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range recombinant human Fas/Fc chimera control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

ANG
AR
CNTF
β-ECGF
EGF
Epo
Fas Ligand
FGF acidic
FGF basic
FGF-4
FGF-5
FGF-6
G-CSF
GM-CSF
gp130
GROα
GROβ
GROγ
HB-EGF
HGF
IFN-γ
IGF-I
IGF-II
IL-1α
IL-1β
IL-1ra
IL-1 RI
IL-1 RII
IL-2
IL-2 Rα
IL-3
IL-3 Rα
IL-4
IL-4 R
IL-5
IL-5 Rα
IL-5 Rβ
IL-6
IL-6 R
IL-7
IL-8
IL-9
IL-10
IL-11
IL-12
IL-13
LAP (TGF-β1)
LIF
M-CSF
MCP-1
MIP-1α
MIP-1β
β-NGF
OSM
PD-ECGF
PDGF-AA
PDGF-AB
PDGF-BB
PTN
RANTES
SCF
SLPI
TGF-α
TGF-β1
TGF-β2
TGF-β3
TGF-β RII
TNF-α
TNF-β
TNF RI
TNF RII
VEGF

Recombinant mouse:

GM-CSF
IL-1α
IL-1β
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-13
LIF
MIP-1α
MIP-1β
SCF
TNF-α

Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF
human TGF-β1
porcine TGF-β1

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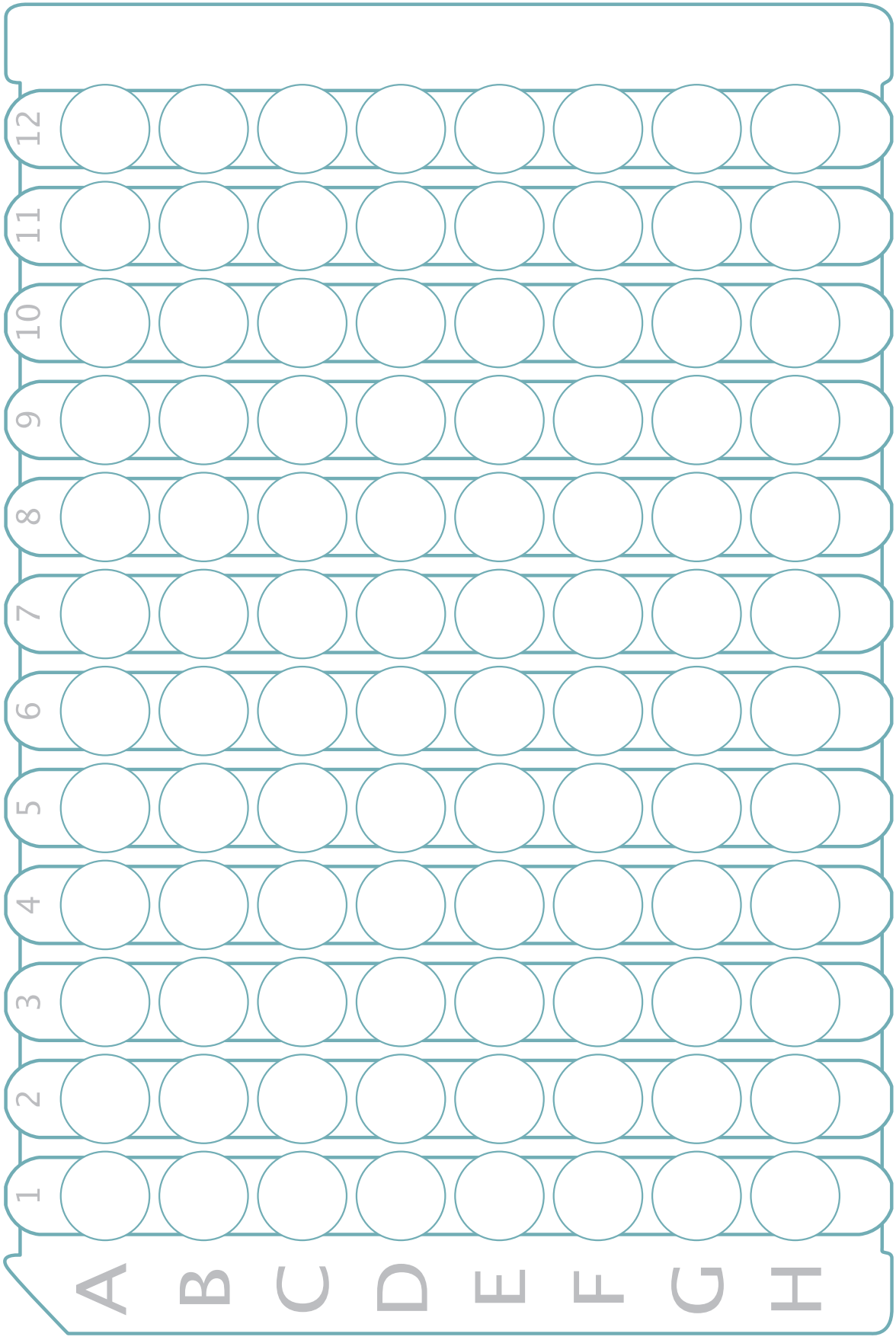
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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