Luminex® Performance Assay

TGF-β Base Kit

Catalog Number LTG000

For the quantitative determination of transforming growth factor beta 1, 2, and 3 (TGF-β1, TGF-β2, and TGF-β3) concentrations in cell culture supernates, serum, plasma, urine, and human milk.

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

Transforming Growth Factor Beta 1, 2, and 3 (TGF-β1, TGF-β2, and TGF-β3) are highly pleiotropic cytokines that are secreted by virtually all cell types. TGF-β molecules are proposed to act as cellular switches that regulate processes such as immune function, proliferation, and epithelial-mesenchymal transition (1-4). Targeted deletions of these genes in mice show that each TGF-β isoform has some non-redundant functions: TGF-β1 is involved in hematopoiesis and endothelial differentiation; TGF-β2 affects development of cardiac, lung, craniofacial, limb, eye, ear, and urogenital systems; and TGF-β3 influences palatogenesis and pulmonary development (2). TGF-β ligands are initially synthesized as precursor proteins that undergo proteolytic cleavage. The mature segments form active ligand dimers via a disulfide-rich core consisting of the characteristic "cysteine knot" (1-4). TGF-β signaling begins with binding to a complex of the accessory receptor betaglycan (also known as TGF-β RIII) and a type II serine/threonine kinase receptor termed TGF-β RII. This receptor then phosphorylates and activates a type I serine/threonine kinase receptor, either ALK-1 or TGF-β RI (also called ALK-5). The activated type I receptor phosphorylates and activates Smad proteins that regulate transcription (3, 5, 6). Use of other signaling pathways that are Smad-independent allows for distinct actions observed in response to TGF-β in different contexts (5).

This kit can be used to assess the levels of three TGF-β molecules. Due to high cross-species homology, human, mouse, rat, and porcine samples can be run in this assay.

Due to a low level of cross-reactivity of the biotinylated anti-TGF-β1 antibody with TGF-β2, multiplexing TGF-β1 and TGF-β2 can result in falsely elevated values for TGF-β2 in some sample types. We found in human serum samples, falsely elevated values for TGF-β2 when multiplexing TGF-β1 and TGF-β2. Similar cross-reactivity was observed in competitor's multiplex assays. For studies using human serum samples, TGF-β1 and TGF-β3, or TGF-β2 and TGF-β3 can be multiplexed; however, careful consideration should be given when deciding to evaluate TGF-β1 and TGF-β2 as a multiplex. Testing with samples from healthy donors found the impact of the cross-reactivity observed became insignificant with human milk, platelet-poor plasma, cell culture supernates and urine samples. It is suggested that each lab evaluate their samples to determine if multiplexing will be problematic (as TGF-β levels will vary with disease state and serum source for media).

Analyte	Catalog Number	Microparticle Region
TGF-β1	LTG100	78
TGF-β2	LTG200	33
TGF-β3	LTG300	6

PRINCIPLE OF THE ASSAY

Luminex® Performance Assay multiplex kits are designed for use with the Luminex® 100™, Luminex 200™, or Bio-Rad® Bio-Plex® dual laser, flow-based sorting and detection analyzers.

Analyte-specific antibodies are pre-coated onto color-coded microparticles. Microparticles, standards and samples are pipetted into wells and the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, biotinylated antibodies specific to the analytes of interest are added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds the biotinylated detection antibodies, is added to each well. A final wash removes unbound Streptavidin-PE and the microparticles are resuspended in buffer and read using the Luminex analyzer. One laser is microparticle-specific and determines which analyte is being detected. The other laser determines the magnitude of the phycoerythrin-derived signal, which is in direct proportion to the amount of analyte bound.

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 fall outside the dynamic range of the assay, further dilute the activated samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, instrumentation, 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 Luminex Performance Assay, the possibility of interference cannot be excluded.
- Discrepancies may exist in values obtained for the same analyte utilizing different technologies.
- Luminex Performance Assays afford the user the benefit of multianalyte analysis in a single complex sample. A single, multipurpose diluent is used to optimize recovery, linearity and reproducibility. Such a multipurpose diluent may not optimize any single analyte to the same degree that a unique diluent selected for analysis of that analyte can optimize conditions. Some performance characteristics may be more variable than those for assays designed specifically for single analyte analysis.
- **Only the analytes listed on the Standard Value Card can be measured with this kit.**

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.
- Protect microparticles and Streptavidin-PE from light at all times to prevent photobleaching.
- For best results, adjust the vacuum strength on the plate washer to between 15 and 40 cm of mercury.

MATERIALS PROVIDED & STORAGE CONDITIONS

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

PART	PART #	DESCRIPTION	STORAGE OF OPENED, DILUTED, OR RECONSTITUTED MATERIAL
TGF-β Standard Cocktail	893244	2 vials of recombinant TGF-β1, 2, and 3 in a buffered protein base with preservatives; lyophilized.	Discard after use. Use a fresh standard for each assay.
Biotin Antibody Diluent 2	895832	5.5 mL of a buffered protein base with preservative.	May be stored for up to 1 month at 2-8 °C.*
Microparticle Diluent 2	895815	6 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-49	895584	21 mL of a buffered protein base with preservatives. *For cell culture supernate/ urine samples.*	
Calibrator Diluent RD6-50	895912	21 mL of a buffered delipidized animal serum with preservatives. *For serum/ plasma/human milk samples.*	
Streptavidin-PE	892525	0.07 mL of a 100-fold concentrated streptavidin-phycoerythrin conjugate with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Microplate	640763	1 filter-bottomed 96-well microplate used as a vessel for the assay.	
Mixing Bottles	895505	2 empty 8 mL bottles used for mixing microparticles with Microparticle Diluent.	
Plate Sealers	640445	4 adhesive foil strips.	
Standard Value Card	749814	1 card listing the Standard reconstitution volume and working standard concentrations for this lot of base kit.	

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

PRECAUTIONS

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

ADDITIONAL REAGENTS REQUIRED

For sample activation:

- Hydrochloric acid (A.C.S. Grade, 12 N)
- Sodium hydroxide (A.C.S. Grade, 10 N)
- HEPES, free acid (Reagent Grade, M.W. 238.3)

OTHER SUPPLIES REQUIRED

- **Luminex Performance Assay analyte-specific kit(s) (see Introduction on page 1).**
- Luminex 100™, Luminex 200™, or BioRad BioPlex Analyzer with X - Y platform.
- Microplate vacuum manifold (Millipore Multiscreen™ Vacuum Manifold, Catalog # MAVM096, or equivalent).
- Pipettes and pipette tips.
- Deionized or distilled water.
- Multi-channel pipette, manifold dispenser, or automated dispensing unit.
- 100 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12″ orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Microcentrifuge.
- **Polypropylene** test tubes for dilution of standards and activation of samples.

SAMPLE COLLECTION AND STORAGE

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

Samples must be assayed immediately after activation. Do not freeze activated samples.

Cell Culture Supernates - Remove particulates by centrifugation and assay (see activation procedure) immediately or aliquot and store unactivated samples at ≤ -70 °C. Avoid repeated freeze-thaw cycles.

Notes: *Animal serum used in the preparation of cell culture media may contain high levels of latent TGF-β1. For best results, do not use animal serum for growth of cell cultures when assaying for TGF-β1 production. If animal serum is used as a supplement in the media, precautions should be taken to prepare the appropriate control and run the control in the assay to determine the baseline concentration of TGF-β1.*

A 10% solution of bovine serum used in cell culture can be expected to contribute typically 100-200 pg/mL of bovine TGF-β2 upon activation. Prepare, activate, and assay an appropriate media control to determine the baseline bovine TGF-β2 in the sample.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature. Centrifuge for 15 minutes at 1000 x g. Remove serum and assay (see activation procedure) immediately or aliquot and store unactivated samples at ≤ -70 °C. Avoid repeated freeze-thaw cycles.

Platelet-poor Plasma* - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. Assay (see activation procedure) immediately or aliquot and store unactivated samples at ≤ -70 °C. Avoid repeated freeze-thaw cycles.

Note: *Grossly hemolyzed or lipemic samples are not suitable for the measurement of TGF-β in this assay.*

***TGF-β1 is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of TGF-β1, platelet-poor plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical and Laboratory Standards Institute (CLSI), result in incomplete removal of platelets from blood. This will cause variable and irreproducible results for assays of factors contained in platelets and released by platelet activation. The recommended plasma collection protocol is designed to minimize platelet degranulation. However, since even the best methods for plasma collection may result in some platelet degranulation on occasion, it is recommended that markers for platelet degranulation be determined in samples containing elevated TGF-β1 levels.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter. Assay immediately (see activation procedure) or aliquot and store unactivated samples at ≤ -70 °C. Avoid repeated freeze/thaw cycles.

Note: *Neat unactivated urine samples exhibit a decrease in TGF-β1 concentration in the first 24 hours of storage (frozen or refrigerated). Care should be taken that all samples are assayed under identical storage conditions and durations.*

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and repeat this process a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

ACTIVATION/NEUTRALIZATION REAGENT PREPARATION

The following solutions may be stored at room temperature for up to one month in polypropylene bottles.

Caution: *Wear protective clothing and safety glasses during preparation or use of these reagents. Refer to the appropriate MSDS prior to use.*

1N HCl (100 mL) - Slowly add 8.33 mL of 12N HCl to 91.67 mL of deionized water. Mix well.

1.2N NaOH/0.5M HEPES (100 mL) - Slowly add 12 mL of 10N NaOH to 75 mL of deionized water. Mix well, and add 11.9 g of HEPES. Mix well, and bring the final volume to 100 mL with deionized water.

SAMPLE ACTIVATION PROCEDURE

To activate latent TGF-β to immunoreactive TGF-β detectable in this assay, follow the activation procedure outlined below for all sample types. Assay samples immediately after neutralization (pH 7.2-7.6). **Use polypropylene test tubes. Do not activate the TGF-β Standard Cocktail.**

1. If samples contain particulates, centrifuge for 2 minutes at 5000 x g before proceeding.
2. Add 20 µL of 1N HCl to 100 µL of sample. Mix well, and incubate for 10 minutes at room temperature.
3. Add 20 µL of 1.2N NaOH/0.5M HEPES. Mix thoroughly.

SAMPLE PREPARATION

Activated cell culture supernates and activated urine samples require a 1:3.57 dilution in Calibrator Diluent RD5-49 after activation. This is a 1:5 final dilution factor. A suggested 1:3.57 dilution is 112 µL of activated/neutralized sample + 288 µL of Calibrator Diluent RD5-49. Mix thoroughly.

Activated serum, plasma, and human milk samples require a 1:10.7 dilution in Calibrator Diluent RD6-50 after activation. This is a 1:15 final dilution factor. A suggested 1:10.7 dilution is 50 µL of activated/neutralized sample + 485 µL of Calibrator Diluent RD6-50. Mix thoroughly.

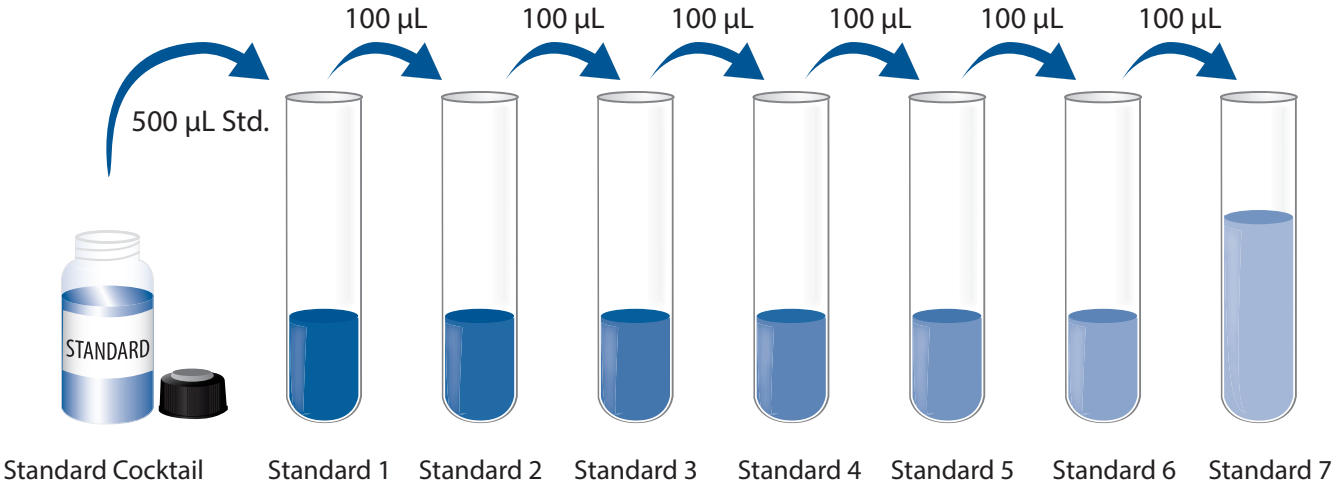
REAGENT PREPARATION

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.

Standard - Reconstitute the Standard Cocktail with Calibrator Diluent RD5-49 (*for cell culture supernate and urine samples*) or Calibrator Diluent RD6-50 (*for serum, plasma, and human milk samples*). Refer to the Standard Card for the reconstitution volume and assigned values. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 500 µL of the reconstituted Standard into the Standard 1 tube. Pipette 200 µL of the appropriate Calibrator Diluent into the remaining tubes. Use Standard 1 to produce a 3-fold dilution series (below). Mix each tube thoroughly before the next transfer. Standard 1 serves as the high standard. The appropriate Calibrator Diluent serves as the blank.



DILUTED MICROPARTICLE COCKTAIL PREPARATION

1. Centrifuge each Microparticle Concentrate vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vials to resuspend the microparticles, taking precautions not to invert the vial.
3. Dilute the Microparticle Concentrates in the mixing bottle provided. The volume of the Microparticle Concentrate listed in the table below is for each analyte (*e.g.* if measuring a full plate, add 50 µL of each Microparticle Concentrate to 5 mL of Microparticle Diluent).

Number of Wells Used	Microparticle Concentrate	+	Microparticle Diluent
96	50.0 µL	+	5.00 mL
72	37.5 µL	+	3.75 mL
48	25.0 µL	+	2.50 mL
24	12.5 µL	+	1.25 mL

Note: *Protect microparticles from light during handling. Diluted microparticles cannot be stored. Prepare microparticles within 30 minutes of use.*

DILUTED BIOTIN ANTIBODY COCKTAIL PREPARATION

1. Centrifuge each Biotin Antibody Concentrate vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vials, taking precautions not to invert the vials.
3. Add 50 µL of each Biotin Antibody Concentrate to the Biotin Antibody Diluent. Mix gently.

STREPTAVIDIN-PE PREPARATION

Use a polypropylene amber bottle or a polypropylene tube wrapped with aluminum foil. Protect Streptavidin-PE from light during handling and storage.

1. Centrifuge the Streptavidin-PE vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial, taking precautions not to invert the vial.
3. Dilute the 100X Streptavidin-PE to a 1X concentration by adding 55 µL of Streptavidin-PE to 5.5 mL of Wash Buffer.

INSTRUMENT SETTINGS

Adjust the probe height setting on the Luminex analyzer to avoid puncturing the membrane. Refer to the instrument manual.

a) Assign the bead region for each analyte being measured (see page 1)
b) 50 events/bead
c) Minimum events: 0
d) Flow rate: 60 µL/minute (fast)
e) Sample size: 50 µL
f) Doublet Discriminator gates at approximately 7500 and 15,500
g) Collect Median Fluorescence Intensity (MFI)

Note: *For the Bio-Rad Bio-Plex analyzer, set the gates at 4300 and 10,000. The CAL2 setting for the Bio-Rad Bio-Plex analyzer should be set at the low RP1 target value.*

ASSAY PROCEDURE

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

Note: *Protect microparticles and Streptavidin-PE from light at all times.*

1. Prepare all reagents, working standards, and samples* as directed in the previous sections.
2. Pre-wet the filter-bottomed microplate by filling each well with 100 µL of Wash Buffer. Remove the liquid through the filter at the bottom of the plate using a vacuum manifold designed to accommodate a microplate.

   Note: *After each final wash cycle and subsequent reagent addition, blot the bottom of the plate with a paper towel to prevent wicking.*
3. Add 50 µL of Standard or activated sample per well.
4. Resuspend the diluted microparticle mixture by inversion or vortexing. Add 50 µL of the microparticle cocktail to each well of the pre-wet filter-bottomed microplate. Securely cover with a foil plate sealer. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12″ orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Using a vacuum manifold device designed to accommodate a microplate, wash by removing the liquid, filling each well with Wash Buffer (100 µL) and removing the liquid again. All of the liquid must be removed through the filter at the bottom of the plate to avoid any loss of microparticles. Complete removal of liquid is essential for good performance. Perform the wash procedure three times.
6. Add 50 µL of diluted Biotin Antibody Cocktail to all wells. Securely cover with a foil plate sealer and incubate for 1 hour at room temperature on the shaker set at 500 ± 50 rpm.
7. Repeat the wash as in step 5.
8. Add 50 µL of diluted Streptavidin-PE to all wells. Securely cover with a foil plate sealer and incubate for 30 minutes at room temperature on the shaker set at 500 ± 50 rpm.
9. Repeat the wash as in step 5.
10. Resuspend the microparticles by adding 100 µL of Wash Buffer to each well. Incubate for 1 minute on the shaker set at 500 ± 50 rpm.
11. Read within 90 minutes using the Luminex or BioRad Analyzer.

*Samples require activation and dilution. See the Sample Activation and Sample Preparation sections.

CALCULATION OF RESULTS

Use the Standard concentrations on the Standard Value Card and calculate 3-fold dilutions for the remaining levels. Average the duplicate readings for each standard and sample and subtract the average blank Median Fluorescence Intensity (MFI).

Create a standard curve for each analyte by reducing the data using computer software capable of generating a five parameter logistic (5-PL) curve-fit.

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

CALIBRATION

This assay is calibrated against highly purified recombinant human TGF-β1, TGF-β2, and TGF-β3 produced at R&D Systems.

SPECIFICITY

This assay recognizes natural and recombinant TGF-βs.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent, assayed, and measured less than 0.5% cross-reactivity unless otherwise noted. The same factors prepared at 50 ng/mL in a mid-range recombinant TGF-β control were assayed for interference. No interference was observed except as noted.

Recombinant human:

6Ckine
Activin A
Activin RIA
Activin RIIA
Activin RIIB
BLC/BCA-1
BMP-2
BMP-3
BMP-3b
BMP-4
BMP-5
BMP-6
BMP-8b
BMP-10
BMP-15
BMP RIA
BMP RIB
BMP RII
CTACK
ENA-78
Eotaxin
Eotaxin-2
Eotaxin-3
Follistatin288
Follistatin300
Follistatin315
GCP-2
GROα
GROβ
GROγ
HCC-1
HCC-4
I-309
IL-8
Inhibin A
Inhibin B
IP-10
I-TAC
LAP
MCP-1
MCP-2
MCP-3
MCP-4
MDC
MIG
MIP-1α
MIP-1β
MIP-1δ
MIP-3α
MIP-3β
MPIF-1
NAP-2
PARC
RANTES
SDF-1α
SDF-1β
TECK
TGF-α
TGF-β RII

Recombinant mouse:

6Ckine
BLC/BCA-1
BMP-3b
BMP RIA
BMP RIB
CRG-2/IP-10
CTACK
Eotaxin
GCP-2
JE/MCP-1
KC
MARC
MCP-5
MDC
MIG
MIP-1α
MIP-1β
MIP-1γ
RANTES
SDF-1α
TARC
TECK

Recombinant rat:

Agrin
MIP-3α

Other recombinants:

porcine IL-8
amphibian TGF-β5

Recombinant human TGF-β1.2 exhibits 4.5% cross-reactivity in this assay.

Recombinant human TGF-β RIII interferes at concentrations ≥ 50 ng/mL.

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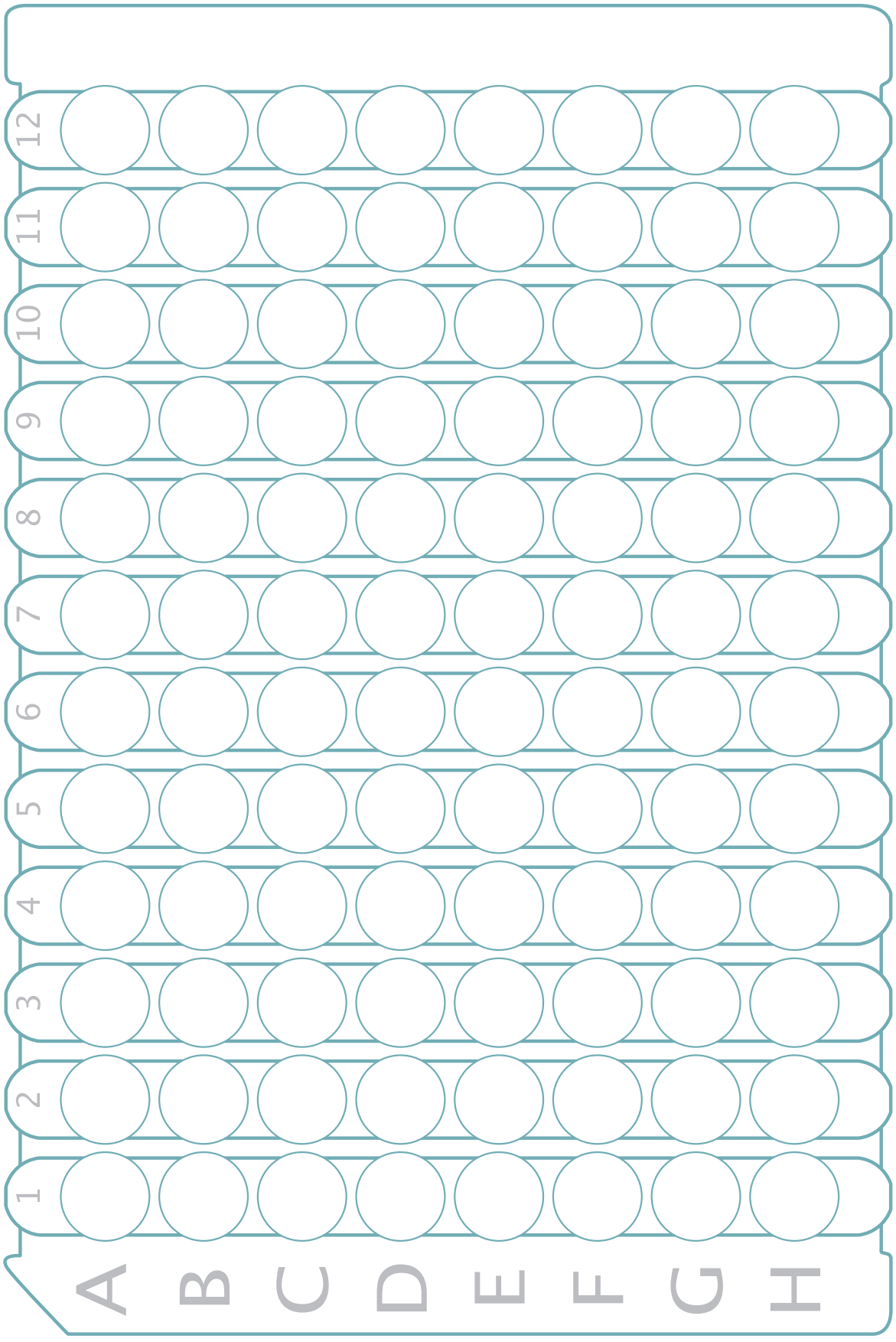
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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