Fluorokine® E

Human Active MMP-13 Fluorescent Assay

Catalog Number F13M00

For the quantitative determination of human active Matrix Metalloproteinase 13 (MMP-13) in cell culture supernates.

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INTRODUCTION

Matrix metalloproteinases (MMPs), also called matrixins, constitute a family of zinc and calcium dependent endopeptidases that function in the breakdown of extracellular matrix (ECM). They play an important role in many normal physiological processes such as embryonic development, morphogenesis, reproduction and tissue remodeling (1). They also participate in many pathological processes such as arthritis, cancer and cardiovascular disease (2). While the amounts of newly synthesized MMPs are regulated mainly at the levels of transcription, the proteolytic activities of existing MMPs are controlled through both the activation of proenzymes or zymogens and the inhibition of active enzymes by endogenous inhibitors, α_2-macroglobulins and tissue inhibitors of metalloproteinases (TIMPs).

As the third collagenase found in humans after MMP-1 (collagenase 1) and MMP-8 (collagenase 2), MMP-13 (collagenase 3) has been proposed to participate in aggrecan degradation associated with osteoarthritis and cleavage of type II collagen in osteoarthritic cartilage explants and in tumor progression and metastasis (3-6). In addition, it can cleave type I, III, IV, IX, X and XIV collagens and fibronectin (4). Therefore, MMP-13 is likely to play a crucial role in the modulation of extracellular matrix degradation and cell-matrix interactions (5). MMP-13 is expressed by several metastatic tumors including breast carcinomas, chondrosarcomas, and head and neck carcinomas, and in degenerative bone diseases including rheumatoid arthritis (6). Wild type and mutant p53, a tumor suppressor gene, differentially regulate MMP-13 gene expression (7). Members of the activator protein-1 and core-binding factor families increase MMP-13 promoter activity in normal, differentiating osteoblasts (6).

MMP-13 is normally secreted as a proenzyme of 452 amino acids. The N-terminal pro-domain (84 residues) contains the cysteine switch motif conserved in MMPs that maintains MMP-13 in a latent state (8). The removal of the pro-domain can be initiated by other MMPs such as MMP-2 and MMP-14, and by plasmin (9). The resulting active enzyme consists of a catalytic domain with a zinc-binding motif conserved in metzincins (10, 11). A short hinge peptide links the catalytic domain to the C-terminal hemopexin-like domain. The C-terminal domain can also be removed from MMP-13 by incubation with plasmin, MMP-2, MMP-14 and *p*-aminophenylmercuric acetate (APMA) (9).

The Fluorokine E Human Active MMP-13 kit combines the specificity of a monoclonal antibody that captures both pro- and active MMP-13 forms and the sensitivity of fluorescence. The kit is designed to measure the levels of endogenous active MMP-13 in cell culture supernates and the MMP-13 in these samples that can be activated by APMA in the assay procedure. The measured MMP-13 activity may reflect the balance of MMP-13 and its inhibitor TIMPs. Therefore, it is recommended that the levels of TIMPs be determined in order to interpret the results properly.

PRINCIPLE OF THE ASSAY

This assay is a fluorimetric assay designed to quantitatively measure enzyme activity. A monoclonal antibody specific for human MMP-13 has been pre-coated onto a black microplate. Standards and samples are pipetted into the wells and any MMP-13 is bound by the immobilized antibody. After washing away any unbound substances, an activation reagent (APMA) is added to standards and selected samples.* Following a wash, a fluorogenic substrate linked to a quencher molecule is added and any active enzyme present will cleave the peptide linker between the fluorophore and the quencher molecule. This cleavage eliminates the distance dependent resonance energy transfer between the fluorophore and the quencher molecule, allowing a fluorescent signal that is proportional to the amount of enzyme activity in the sample.

FL = Fluorophore
Q = Quencher

Capture sample/standard, Activate   Add quenched fluorogenic substrate   Cleavage of peptide linker by active enzyme
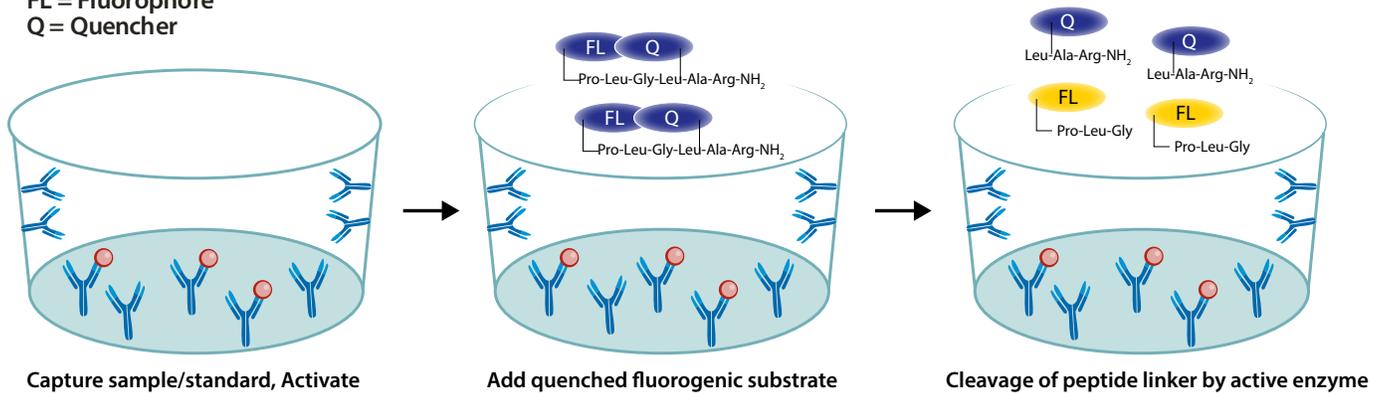
*This kit is designed to measure the levels of both endogenous active MMP-13 in cell culture supernates and the MMP-13 in these samples that can be activated by APMA in the assay procedure.

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 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 Fluorokine E Immunoassay, the possibility of interference cannot be excluded.
- Relative fluorescence units (RFU) may differ among fluorimeters. The Human Active MMP-13 Fluorokine E Assay was optimized using a Molecular Devices *fMax*™ fluorimeter. Other instruments may require settings to be adjusted.

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.
- A humidified environment can be made by placing moist paper towels in a sealed container.

PRECAUTIONS

Some components of this kit contain sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The APMA provided with this kit is a mercury containing compound. The total amount of mercury in this kit is 20 mg. Dispose of according to local, state, and federal regulations.

The APMA and Substrate provided with this kit are hazardous components containing DMSO. Wear gloves and protective clothing when handling these materials. Dispose of according to local, state, and federal regulations.

Some components in this kit contain ProClin® 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.

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 Active MMP-13 Microplate	891033	96 well black polystyrene microplate (6 strips of 16 wells) coated with monoclonal antibody specific for human MMP-13.	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.*
MMP-13 Standard	891034	3 vials of recombinant human pro-MMP-13 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a fresh standard for each assay.
Calibrator Diluent RD5-25	895356	21 mL of a buffered protein base with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Reagent Diluent 2	895357	2 vials (22 mL/vial) of a Tris-HCl buffer with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
***p*-Aminophenylmercuric Acetate (APMA)**	895327	200 µL of a stock solution of 0.5 M APMA in DMSO.	Discard after use. Prepare fresh for each assay. Store stock solutions for up to 1 month at 2-8 °C.*
Substrate	895292	400 µL of a stock solution of 1 mM fluorogenic substrate in DMSO.	
Plate Sealers	N/A	8 adhesive strips.	

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

OTHER SUPPLIES REQUIRED

• ***f**Max* fluorimeter set with the following parameters: excitation wavelength set to 320 nm or 340 nm and emission wavelength set to 405 nm; endpoint mode; 1 x 20 mS integration time; plate speed = 6, or the equivalent.

• Pipettes and pipette tips.

• Deionized or distilled water.

• Squirt bottle, manifold dispenser, or automated microplate washer.

• 500 mL graduated cylinder.

• 37 °C incubator.

• Humidified environment (*e.g.* sealable bag with moist paper towels or humidified chamber)

• Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.

• **Polypropylene** test tubes for dilution of standards and samples.

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.

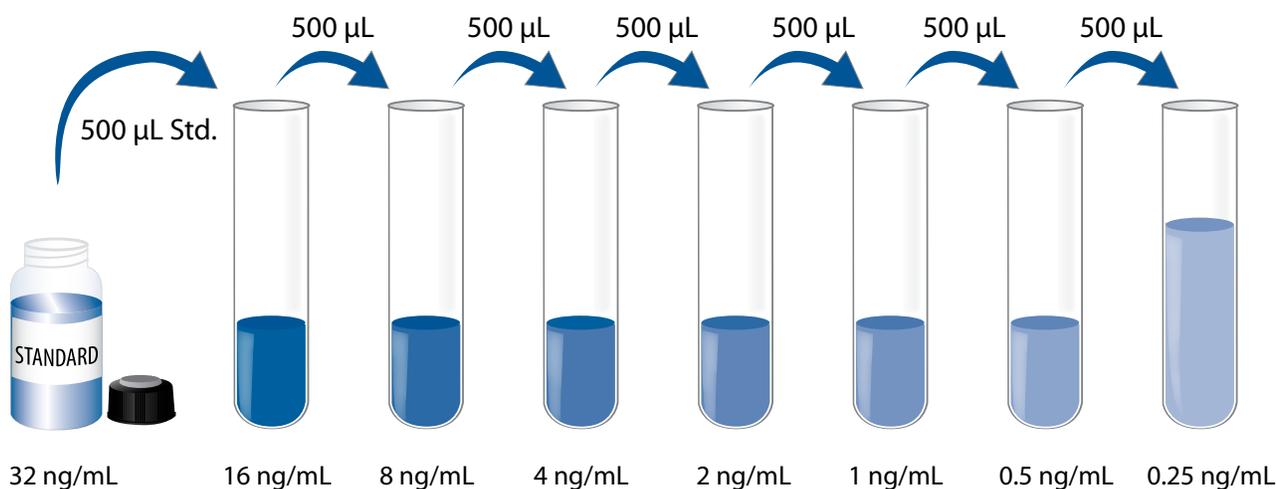**Note:** *Serum and plasma samples have not been validated for use in this assay.*

REAGENT PREPARATION

Bring all reagents to room temperature before use.
Substrate and APMA may be warmed to 37 °C.

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.

MMP-13 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the MMP-13 Standard with deionized or distilled water. This reconstitution produces a stock solution of 32 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 500 µL of Calibrator Diluent RD5-25 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 16 ng/mL standard serves as the high standard. The Calibrator Diluent RD5-25 serves as the zero standard (0 ng/mL).

| 500 µL Std. | 500 µL | 500 µL | 500 µL | 500 µL | 500 µL | 500 µL |

| 32 ng/mL | 16 ng/mL | 8 ng/mL | 4 ng/mL | 2 ng/mL | 1 ng/mL | 0.5 ng/mL | 0.25 ng/mL |

REAGENT PREPARATION *CONTINUED*

***p*-aminophenylmercuric acetate (APMA)** - The APMA Solution should be prepared within 15 minutes of use. Tap the vial gently to dislodge any APMA in the vial cap. Prepare only the amount needed for each assay (200 µL of the diluted APMA is needed per well). Dilute APMA 168-fold with Reagent Diluent 2. Solution will appear cloudy and contain a precipitate; vortex well. Example dilutions are listed in the table below. Discard any unused diluted APMA. Prepare fresh APMA for each assay.

Note: *Prepare only the amount of APMA needed for standard wells and any desired sample wells to be activated.*

APMA Dilution

Number of Wells	APMA Stock	+	Reagent Diluent 2	Total APMA
32	42 µL	+	6.96 mL	7 mL
64	83 µL	+	13.92 mL	14 mL
96*	135 µL	+	22.5 mL	22.64 mL*

When activating a full plate, it is recommended to spike the stock solution into the full bottle (22.5 mL) of Reagent Diluent 2. Label the bottle "APMA" to avoid reagent mixup.

Substrate Solution - Substrate solution should be prepared within 15 minutes of use. Protect from light prior to use. Tap vial gently to dislodge any substrate from the vial cap. Prepare only the amount needed for each assay (200 µL of the diluted substrate is needed per well). Dilute Substrate stock 61-fold with Reagent Diluent 2. Example dilutions are listed in the table below. Discard any unused diluted substrate. Prepare fresh Substrate for each assay.

Substrate Dilution

Number of Wells	Substrate Stock	+	Reagent Diluent 2	Total Substrate
32	115 µL	+	6.89 mL	7 mL
64	230 µL	+	13.78 mL	14 mL
96*	375 µL	+	22.5 mL	22.88 mL*

When assaying a full plate, it is recommended to spike the stock solution into the full bottle (22 mL) of Reagent Diluent 2. Label the bottle "Substrate" to avoid reagent mixup.

For research use only. Not for use in diagnostic procedures.

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 200 μL of Standard, sample, or control per well. Cover with the adhesive strip provided. Incubate for 3 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.

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 200 μL of diluted APMA to all standard wells and any desired sample wells. Cover with the adhesive strip provided. Incubate for 1 hour* at **37 °C in a humidified environment. Protect from light.**

   Note: *The addition of APMA will activate any potentially active forms of MMP-13 present in the sample. To measure endogenous levels of active MMP-13 in samples, do not add APMA to the sample wells. Add 200 μL Reagent Diluent 2 to these sample wells instead. APMA must always be added to the standard wells.*

6. Repeat the aspiration/wash as in step 4.

7. Add 200 μL of Substrate to each well. Cover with a new adhesive strip. **Protect the plate from light within 10 minutes of Substrate addition.** Incubate for 17-20 hours at **37 °C in a dark, humidified environment.**

   Note: *Exposure of Substrate to light for greater than 10 minutes may cause the Substrate to degrade. It is recommended that the addition of Substrate be performed in a low light environment and be completed and protected completely from light within 10 minutes.*

8. Determine the relative fluorescence units (RFU) of each well using a fluorescence plate reader set with the following parameters: excitation wavelength set to 320 nm and emission wavelength set to 405 nm; endpoint mode; 1 x 20 mS integration time; plate speed=6.

*Incubation time with APMA is critical and should not exceed 1 hour.
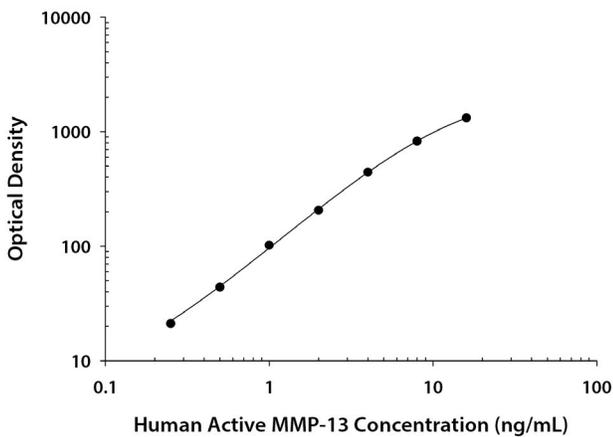
CALCULATION OF RESULTS

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 human active MMP-13 concentrations versus the log of the RFU 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)	RFU	Average	Corrected
0	17.3	17.4	—
	17.6		
0.25	37.9	38.5	21.1
	39.1		
0.5	60.0	61.3	43.9
	62.6		
1	117	119	102
	121		
2	211	223	206
	236		
4	443	460	443
	477		
8	812	845	828
	878		
16	1293	1336	1319
	1379		

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 components.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	0.61	2.84	7.59	0.66	2.81	7.55
Standard deviation	0.03	0.12	0.39	0.05	0.17	0.49
CV (%)	4.9	4.2	5.1	7.6	6.0	6.5

RECOVERY

The recovery of active human MMP-13 spiked to levels throughout the range of the assay. Samples were activated during the assay with the addition of APMA and the recovery of active MMP-13 was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	102	91-111%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human active MMP-13 were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay. All samples were activated during the assay with the additon of APMA.

		Cell culture media (n=4)	HT1080 Cells (n=1)
1:2	Average % of Expected	101	100
	Range (%)	93-113	——
1:4	Average % of Expected	105	99
	Range (%)	99-114	——
1:8	Average % of Expected	108	95
	Range (%)	100-113	——
1:16	Average % of Expected	102	98
	Range (%)	99-105	——

SENSITIVITY

Forty-one assays were evaluated and the minimum detectable dose (MDD) of human active MMP-13 ranged from 0.003-0.015 ng/mL. The mean MDD was 0.008 ng/mL.

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

CALIBRATION

This assay is calibrated against highly purified NS0-expressed recombinant human pro-MMP-13 produced at R&D Systems.

SAMPLE VALUES

Cell Culture Supernates:

HT1080 human fibrosarcoma cells were cultured in DMEM supplemented with 10% fetal calf serum. The cells were cultured for 4 days unstimulated or stimulated with 50 ng/mL PMA and 500 ng/mL calcium ionomycin. Aliquots of the cell culture supernates were removed and assayed for levels of human active MMP-13 (APMA activated) and endogenous active MMP-13 (no APMA).

Condition	MMP-13 (APMA activated)	endogenous active MMP-13 (no APMA)
Unstimulated	ND	ND
Stimulated	38.8 ng/mL	ND

ND=Non-detectable

U2OS human osteosarcoma cells were grown to 100% confluence in McCoy's 5a media with 15% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin sulfate. Aliquots of the cell culture supernates were removed and assayed for levels of human active MMP-13 (APMA activated) and endogenous human active MMP-13 (no APMA).

Cell Line	MMP-13 (APMA activated)	endogenous active MMP-13 (no APMA)
U2OS	0.371 ng/mL	ND

ND=Non-detectable

SPECIFICITY

This assay recognizes natural and recombinant human active MMP-13.

The factors listed below were prepared at 160 ng/mL in Calibrator Diluent and assayed for cross-reactivity. No significant cross-reactivity was observed.

Recombinant human:
MMP-1
MMP-2
MMP-3
MMP-7
MMP-8
MMP-9
MMP-10
MMP-12 Hemopexin
TIMP-1
TIMP-2

MMP-13/TIMP-1 complexes were formed at 2-8 °C overnight by mixing the human active MMP-13 (APMA activated rhMMP-13) with a 10X molar excess of rhTIMP-1. The control was human active MMP-13 alone. Samples were diluted to different concentrations and evaluated for activity.

No active MMP-13 was detected in the active MMP-13/TIMP-1 complex while 100% of the active MMP-13 control was detected.
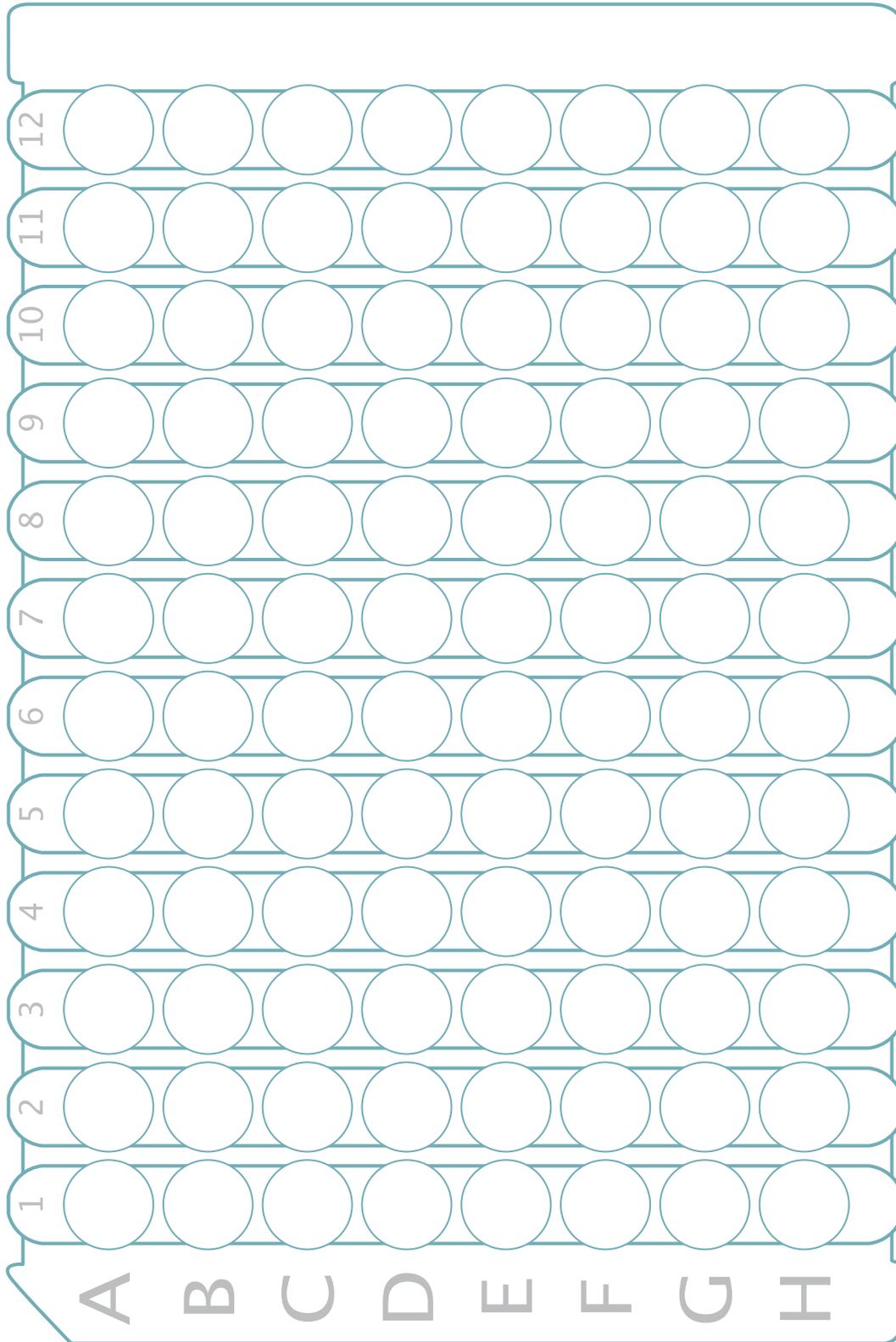
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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

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14   For research use only. Not for use in diagnostic procedures.