MSD® MULTI-SPOT Assay System

Muscle Injury Panel 2 (rat) Kit

1-Plate Kit	K15180C-1
5-Plate Kit	K15180C-2
25-Plate Kit	K15180C-4

MSD Toxicology Assays

Muscle Injury Panel 2 (rat) Kit

TIMP-1, CK

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

Tissue inhibitor of metalloproteinases I (TIMP-1) is an endogenous inhibitor of matrix metalloproteinases.[1] TIMPs have been implicated in direct regulation of cell growth and apoptosis.[2] TIMP-1 plays a role in pathologic processes associated with rheumatoid arthritis[3] and cardiovascular diseases.[4] It can bind to both activated and latent forms of MMP-9.[5] TIMP-1 is associated with inflammation and vasculitis, both of which can result in muscle damage.

Creatine kinase (CK) is an enzyme that catalyzes the rapid regeneration of ATP. It allows muscle contraction by transferring the phosphoryl group from phosphocreatine to ADP, resulting in creatine and ATP.[6] In rats, CK activity is present in heart, brain, skeletal and smooth muscle.[7] Increased CK concentration is indicative of muscle injury.

Principle of the Assay

MSD toxicology assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. The assays in the Muscle Injury Panel 2 (rat) Kit are sandwich immunoassays (Figure 1). MSD provides a plate pre-coated with capture antibodies. The user adds the sample and a solution containing detection antibodies conjugated with electrochemiluminescent labels (MSD SULFO-TAG™) over the course of one or more incubation periods. Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the detection antibodies by the bound analytes completes the sandwich. The user adds an MSD buffer that provides the appropriate chemical environment for electrochemiluminescence and loads the plate into an MSD instrument where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures the intensity of emitted light to provide a quantitative measure of analytes in the sample.

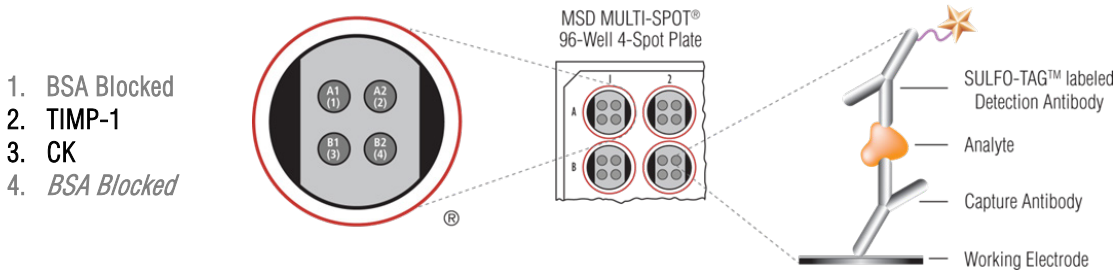


Figure 1. Spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing records.

Reagents Supplied

Product Description	Storage	Quantity per Kit K15180C-1	K15180C-2	K15180C-4
MULTI-SPOT 96-Well 4-Spot Muscle Injury Panel 2 (rat) Plate N45180A-1	2–8°C	1 plate	5 plates	25 plates
SULFO-TAG Anti-rat TIMP-1 Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-rat CK Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
Muscle Injury Panel 2 (rat) Calibrator Blend (20X)	≤-70°C	1 vial (20 µL)	5 vials (20 µL ea)	25 vials (20 µL ea)
Diluent 5 R52BA-4 (5 mL), R52BA-5 (25 mL)	≤-10°C	1 bottle (5 mL)	1 bottle (25 mL)	5 bottles (25 mL ea)
Diluent 11 R55BA-3 (50 mL)	≤-10°C	1 bottle (50 mL)	2 bottles (50 mL ea)	10 bottles (50 mL ea)
Blocker D-R[2] (10%)	≤-10°C	1 vial (0.2 mL)	1 vial (1.0 mL)	5 vials (1.0 mL ea)
Blocker A Kit R93AA-2 (250 mL)	RT	1 bottle (250 mL)	1 bottle (250 mL)	5 bottles (250 mL ea)
Read Buffer T (4X) R92TC-3 (50 mL)	RT	1 bottle (50 mL)	1 bottle (50 mL)	5 bottles (50 mL ea)

Required Material and Equipment (not supplied)

- ❑ Deionized water for diluting concentrated buffers
- ❑ Appropriately sized tubes for reagent preparation
- ❑ Microcentrifuge tubes for preparing serial dilutions
- ❑ Phosphate buffered saline plus 0.05% Tween-20 (PBS-T) for plate washing
- ❑ Liquid handling equipment for desired throughput, capable of dispensing 10 to 150 µL/well into a 96-well microtiter plate
- ❑ Plate washing equipment: automated plate washer or multichannel pipette
- ❑ Adhesive plate seals
- ❑ Microtiter plate shaker

[1] SULFO-TAG–conjugated detection antibodies should be stored in the dark.
[2] Blocker D-R can tolerate at least 5 freeze-thaw cycles. Alternatively, an aliquot of Blocker D-R can be stored at 2–8°C up to 1 month.

Safety

Use safe laboratory practices and wear gloves, safety glasses, and lab coats when handling kit components. Handle and dispose of all hazardous samples properly in accordance with local, state, and federal guidelines.

Reagent Preparation

Bring all reagents to room temperature. Thaw the stock calibrator on ice.

> **Important:** Upon first thaw, separate Diluent 5 and Diluent 11 into aliquots appropriate for the size of your needs before refreezing.

Prepare Blocker A Solution

Follow instructions included with the Blocker A kit.

Prepare Standards

MSD supplies a blended calibrator for the Muscle Injury Panel 2 (rat) Kit at 20-fold higher concentration than the recommended highest standard. We recommend a 7-point standard curve with 4-fold serial dilution steps and a zero calibrator blank. Signals from the blank should be excluded when generating the curve. Thaw the stock calibrator and keep on ice, then add to diluent at room temperature to make the standard curve solutions. For the actual concentration of each calibrator in the blend, refer to the certificate of analysis (C of A) supplied with the kit. You may also find a copy of the lot-specific C of A at www.mesoscale.com.

To prepare 7 standard solutions plus a zero calibrator blank for up to 4 replicates:

1) Prepare the highest standard by adding 10 µL of stock calibrator to 190 µL of Diluent 11. Mix well.
2) Prepare the next standard by transferring 50 µL of the highest standard to 150 µL of Diluent 11. Mix well. Repeat 4-fold serial dilutions 5 additional times to generate 7 standards.
3) Use Diluent 11 as the blank.

Dilute Samples

For rat serum and plasma samples, MSD recommends a 20-fold dilution in Diluent 11; however, you may adjust dilution factors for the sample set under investigation. Samples collected via cardiac puncture may require 100-fold dilution or higher.

To dilute sample 20-fold, add 10 µL of sample to 190 µL of Diluent 11.

Prepare Detection Antibody Solution

MSD provides each detection antibody in a 50X stock solution. The working detection antibody solution is 1X.

For 1 plate, combine:

- ❑ 60 µL of 50X SULFO-TAG Anti-rat TIMP-1 Antibody
- ❑ 60 µL of 50X SULFO-TAG Anti-rat CK Antibody
- ❑ 90 µL of Blocker D-R
- ❑ 2790 µL of Diluent 5

Note: You may omit detection antibody for any analyte not being measured; add 60 µL of Diluent 5 for each omitted antibody.

Prepare Read Buffer

MSD provides Read Buffer T as a 4X stock solution. The working solution is 1X.

For 1 plate, combine:

- ❑ 5 mL of Read Buffer T (4X)
- ❑ 15 mL of deionized water

You may prepare diluted read buffer in advance and store it at room temperature in a tightly sealed container.

Prepare MSD Plate

MSD plates are pre-coated with capture antibodies (Figure 1) and exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies. Plates can be used as delivered; no additional preparation (e.g., pre-wetting) is required.

Protocol

1. **Add Blocker A Solution:** Add 150 µL of Blocker A solution to each well. Seal the plate with an adhesive plate seal and incubate for 1 hour with vigorous shaking (300–1000 rpm) at room temperature.

2. **Wash and Add Sample or Calibrator:** Wash the plate 3 times with 300 µL/well of PBS-T. Add 25 µL of calibrator or diluted sample per well. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.

   You may prepare detection antibody solution during incubation.

3. **Wash and Add Detection Antibody Solution:** Wash the plate 3 times with 300 µL/well of PBS-T. Add 25 µL of 1X detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.

   You may prepare diluted read buffer during incubation.

4. **Wash and Read:** Wash the plate 3 times with 300 µL/well of PBS-T. Add 150 µL of 1X Read Buffer T to each well. Analyze the plate on the SECTOR Imager. No incubation in read buffer is required before reading the plate.

Notes

Shaking the plate typically accelerates capture at the working electrode.

You may keep excess diluted read buffer in a tightly sealed container at room temperature for later use.

Bubbles introduced when adding read buffer will interfere with imaging of the plate and produce unreliable data. Use reverse pipetting technique to avoid creating bubbles.

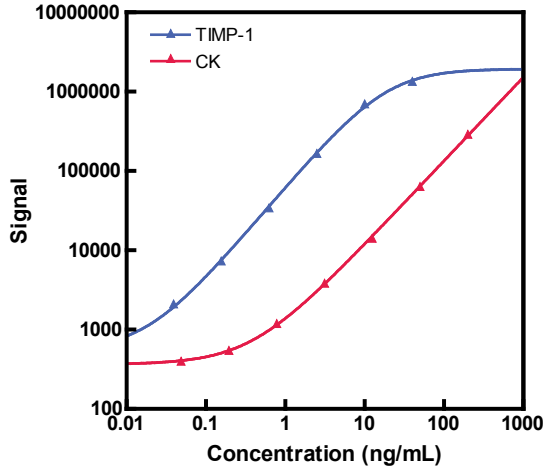
Due to the varying nature of each research application, you should assess assay stability before allowing plates to sit with read buffer for extended periods.

Curve Fitting

MSD DISCOVERY WORKBENCH® software uses least-squares fitting algorithms to generate a standard curve that will be used to calculate the concentration of analyte in the samples. The assays have a wide dynamic range (3–4 logs) that allows accurate quantification without the need for dilution in many cases. The software uses a 4-parameter logistic model (or sigmoidal dose-response) and includes a $1/Y^2$ weighting function. The weighting function is important because it provides a better fit of data over a wide dynamic range, particularly at the low end of the standard curve.

Typical Data

The following standard curves illustrate the dynamic range of the assay. Actual signals will vary. Best quantification of unknown samples will be achieved by generating a standard curve for each plate using a minimum of 2 replicates of standards. For each kit lot, refer to the C of A for the actual concentration of the calibrator.



TIMP-1		
Conc. (ng/mL)	Average Signal	%CV
0	342	4.3
0.0098	799	2.9
0.039	2112	3.4
0.16	7310	2.7
0.63	34 295	1.9
2.5	166 070	0.6
10	699 702	5.4
40	1 344 226	1.6

CK		
Conc. (ng/mL)	Average Signal	%CV
0	302	1.6
0.049	399	6.6
0.20	547	0.0
0.78	1194	2.8
3.1	3852	3.5
13	14 128	0.7
50	63 614	0.0
200	290 284	1.6

Sensitivity

The lower limit of detection (LLOD) is a calculated concentration based on a signal 2.5 standard deviations above the background (zero calibrator blank). The LLOD shown below was calculated based on 20 runs.

	TIMP-1	CK
Average LLOD (ng/mL)	0.014	0.093
LLOD Range (ng/mL)	0.011–0.024	0.056–0.16

Precision

Rat serum-based controls (controls 1 and 2) and diluent-based control (control 3) were measured using a minimum of 2 replicates on 17 runs over 9 days.

Average intra-run %CV is the average %CV of the control replicates on an individual run.

Inter-run %CV is the variability of controls across 17 runs.

	Control	Runs	Average Conc. (ng/mL)	Average Intra-run %CV	Inter-run %CV
TIMP-1	Control 1	17	11	4.8	8.8
	Control 2	17	0.36	5.7	9.4
CK	Control 1	17	61	4.5	13.2
	Control 2	17	9.2	3.5	15.1
	Control 3	17	2.5	4.6	7.6

Spike Recovery

Normal rat serum, EDTA plasma, and heparin plasma samples were diluted 20-fold then spiked with calibrators at multiple levels throughout the range of the assay. The average percent recovery shown below was calculated from samples with values above the LLOD. % Recovery=measured/expected∗100

	TIMP-1			CK		
Sample Type	Spike Conc. (ng/mL)	Average % Recovery	% Recovery Range	Spike Conc. (ng/mL)	Average % Recovery	% Recovery Range
Serum (N=6)	1.2	110	93–122	5.9	109	101–115
	3.6	111	101–125	18	110	93–118
	11	115	97–127	53	109	89–116
EDTA Plasma (N=4)	1.2	108	93–124	5.9	112	102–126
	3.6	112	103–122	18	111	104–119
	11	115	101–124	53	109	101–116
Heparin Plasma (N=3)	1.2	107	96–115	5.9	106	97–110
	3.6	109	98–125	18	107	94–114
	11	112	102–129	53	103	95–108

Dilution Linearity

To assess linearity, normal rat serum and plasma samples were diluted 10-fold, 20-fold, 40-fold, 80-fold, and 160-fold before testing. Percent recovery at each dilution was calculated by dividing the measured concentration by the expected concentration, i.e., the concentration of the previous dilution. The average percent recovery shown below was calculated from samples with values above the LLOD. % Recovery=measured∗/expected∗100

		TIMP-1		CK	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=4)	20	102	98–107	110	100–134
	40	94	88–97	97	91–103
	80	99	94–102	90	80–107
	160	103	101–104	89	78–98
EDTA Plasma (N=4)	20	99	93–104	137	104–185
	40	94	91–99	105	95–121
	80	99	90–105	104	85–130
	160	103	99–110	91	64–113

Specificity

To assess specificity of the detection antibodies, the Muscle Injury Panel 2 (rat) was run using blended calibrators with individual detection antibodies and using blended detction antibodies with individual calibrators (2.5 ng/mL TIMP-1; 13 ng/mL CK). No signficant cross-reactivity (<0.5%) was observed.

In most cells, the CK enzyme consists of 2 subunits, which can be either B (brain type) or M (muscle type). This results in 3 isoenzymes: CK-MM, CK-BB, and CK-MB. The MSD CK assay recognizes recombinant rat CK-MM, recombinant human CK-MM, and recombinant human CK-MB. It does not cross-react with recombinant human CK-BB. The assay measures 100-fold higher CK concentrations in rat quadriceps tissue homogenate than in rat cardiac tissue homogenate.

Stability

Kit components were tested for freeze–thaw stability. Results (not shown) demonstrated that blended calibrator, controls, Diluent 5, and Diluent 11 can go through 5 freeze–thaw cycles without significantly affecting assay performance.

Tested Samples

Serum, EDTA plasma, and heparin plasma samples were collected from normal Sprague-Dawley rats, diluted 20-fold, and tested with the Muscle Injury Panel 2 (rat). Median and range of concentrations for each sample set are displayed below. Concentrations are corrected for sample dilution.

Sample Type	Statistic	TIMP-1	CK
Serum	Median (ng/mL)	11	126
	Range (ng/mL)	6.7–16	1.4–2304
	Number of Samples	12	12
EDTA Plasma	Median (ng/mL)	6.6	60
	Range (ng/mL)	5.0–11	17–698
	Number of Samples	6	6
Heparin Plasma	Median (ng/mL)	8.6	179
	Range (ng/mL)	7.6–12	72–277
	Number of Samples	4	4

Assay Components

Calibrators

The assay calibrator blend uses the following recombinant proteins:

Rat TIMP-1 (residues 24-217) expressed in murine myeloma cells

Human CK-MM with identical amino acid sequence to that of the native enzyme expressed in *Pichia pastoris*

Antibodies

	Source Species	
Analyte	MSD Capture Antibody	MSD Detection Antibody
TIMP-1	Mouse Monoclonal	Goat Polyclonal
CK	Mouse Monoclonal	Mouse Monoclonal

References

1. Chiro R, et al. Novel functions of TIMPs in cell signaling. Cancer Met Rev. 2006;1:99-113.
2. Stetler-Stevenson WG. Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities. Sci Signal. 2008;1:re6.
3. Giannelli G, et al. MMP-2, MMP-9, TIMP-1 and TIMP-2 levels in patients with rheumatoid arthritis and psoriatic arthritis. Clin Exp Rheumatol. 2004;2293:335-8.
4. Sundström J, et al. Relations of plasma total TIMP-1 levels to cardiovascular risk factors and echocardiographic measures: the Framingham heart study. Eur Heart J. 2004:25:1509-16.
5. Roderfeld M, Graf J, et al. Latent MMP-9 is bound to TIMP-1 before secretion. Biol Chem. 2007;388:1227-34.
6. Matthews RT, et al. Neuroprotective effects of creatine and cyclocreatine in animal models of Huntington's disease. J Neurosci. 2008;18: 156-63.
7. Jacobus WE, Lehninger AL. Creatine kinase of rat heart mitochondria. Coupling of creatine phosphorylation to electron transport. J Biol Chem. 1973;248:4803-10.

Summary Protocol

MSD 96-well MULTI-SPOT Muscle Injury Panel 2 (rat) Kit

MSD provides this summary protocol for your convenience. Please read the entire detailed protocol prior to performing the Muscle Injury Panel 2 (rat) assays.

Sample and Reagent Preparation

Bring all reagents to room temperature and thaw the calibrator on ice.

Prepare Blocker A solution.

Prepare 7 standard solutions using the supplied calibrator:

- Dilute the stock calibrator blend 20-fold in Diluent 11.
- Perform a series of 4-fold dilution steps and prepare a zero calibrator blank.

Dilute samples 20-fold in Diluent 11 before adding to the plate.

Prepare combined detection antibody solution by diluting each stock detection antibody 50-fold in Diluent 5 containing Blocker D-R.

Prepare 1X Read Buffer T by diluting stock 4X Read Buffer T 4-fold with deionized water.

Step 1: Add Blocker A Solution

Add 150 µL/well of Blocker A Solution.

Incubate at room temperature with vigorous shaking (300–1000 rpm) for 1 hour.

Step 2: Wash and Add Sample or Calibrator

Wash plate 3 times with 300 µL/well of PBS-T.

Add 25 µL/well of calibrator or diluted sample.

Incubate at room temperature with vigorous shaking (300–1000 rpm) for 2 hours.

Step 3: Wash and Add Detection Antibody Solution

Wash plate 3 times with 300 µL/well of PBS-T.

Add 25 µL/well of 1X detection antibody solution.

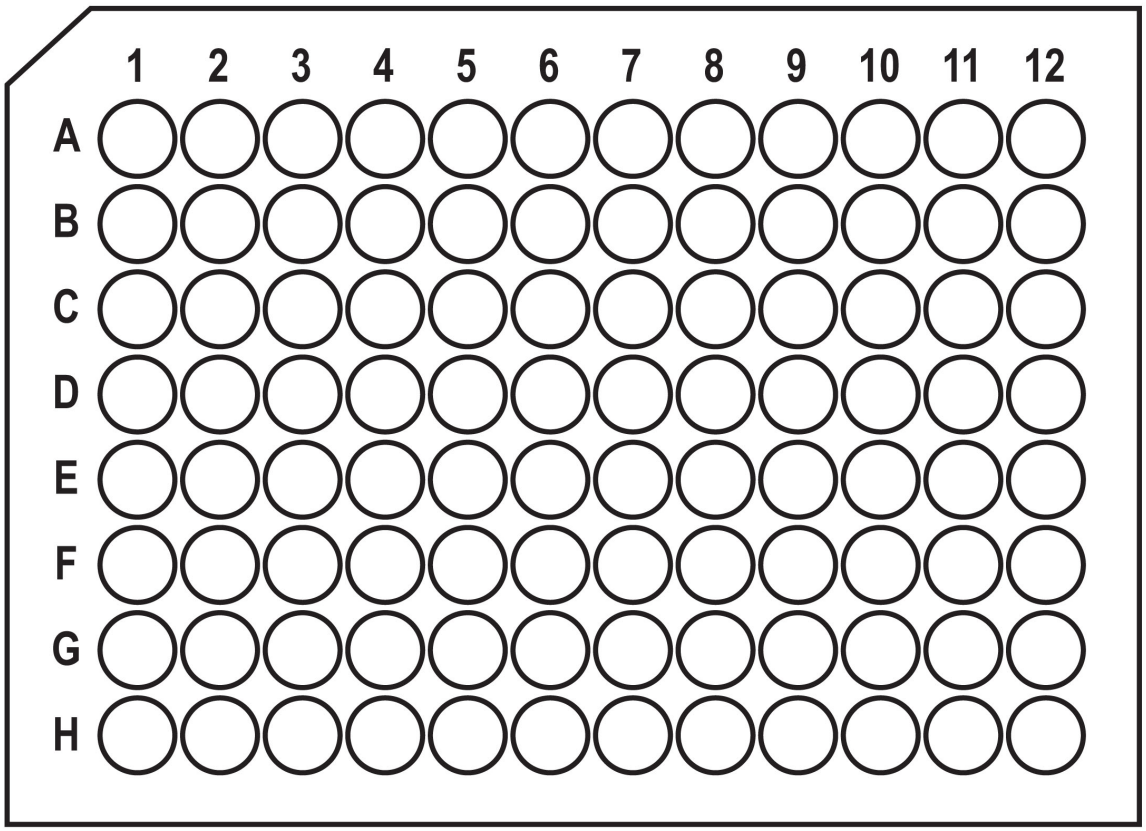
Incubate at room temperature with vigorous shaking (300–1000 rpm) for 2 hours.

Step 4: Wash and Read Plate

Wash plate 3 times with 300 µL/well of PBS-T.

Add 150 µL/well of 1X Read Buffer T.

Analyze plate on SECTOR Imager.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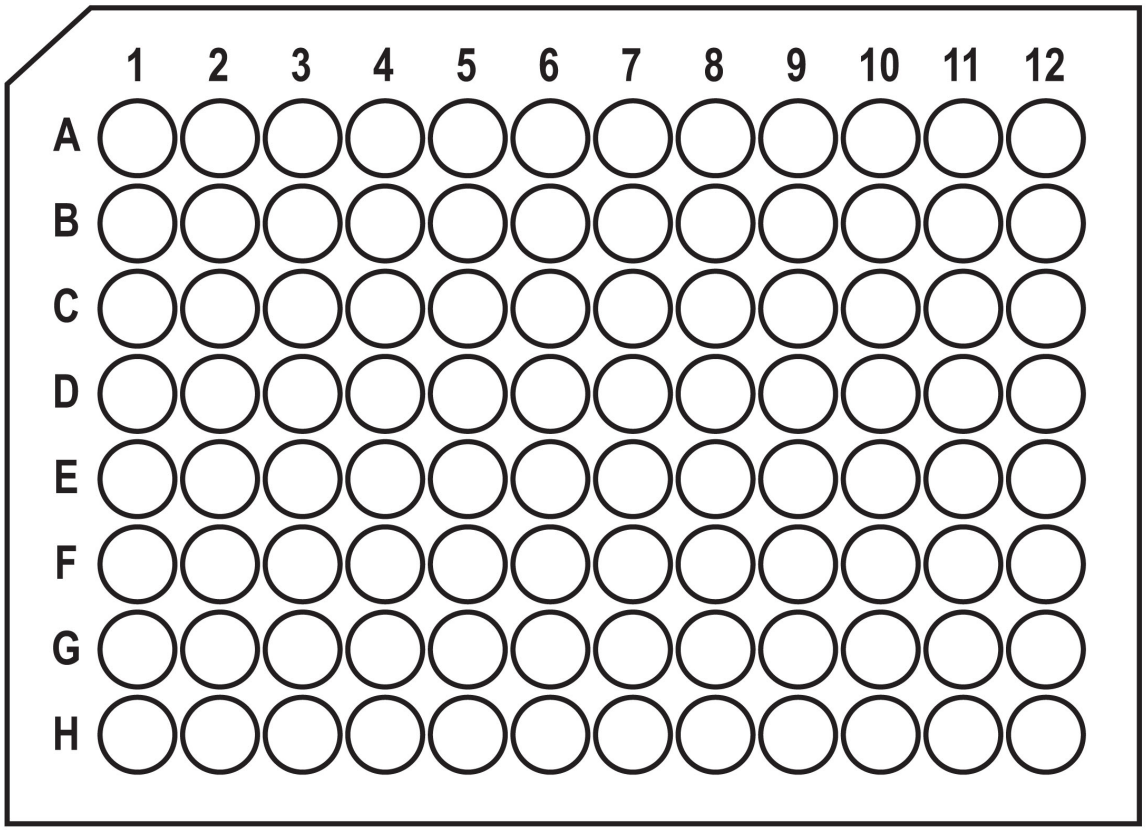
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