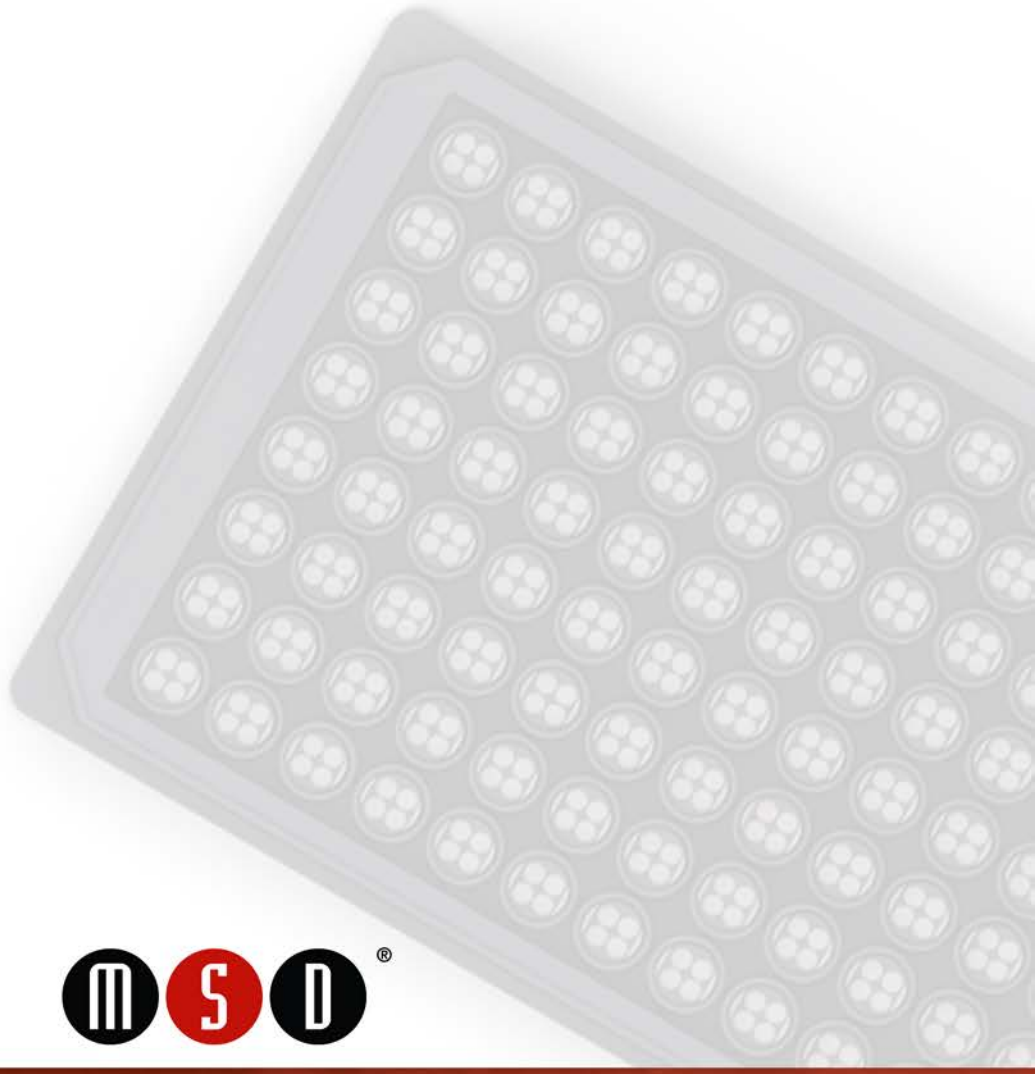
MSD® MULTI-SPOT Assay System

Inflammation Panel 3 (cyno) Kit

1-Plate Kit	K15191D-1
5-Plate Kit	K15191D-2
25-Plate Kit	K15191D-4



MSD Toxicology Assays

Inflammation Panel 3 (cyno) Kit
MCP-1, NGAL, TIMP-1

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

MESO SCALE DISCOVERY®
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Ordering Information

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Introduction

The MSD Inflammation Panel 3 is designed for the quantitative measurements of MCP-1, NGAL, and TIMP-1 in cynomolgus (cyno) monkey serum and plasma.

Monocyte chemoattractant protein-1 (MCP-1) is a 13 kDa chemotactic cytokine[1] that induces leukocyte migration and activation to the site of infection.[2] MCP-1 is produced predominantly by macrophages and endothelial cells and is a potent chemotactic factor for monocytes.[3-5] MCP-1 expression is linked to diseases such as atherosclerosis, multiple sclerosis, and rheumatoid arthritis. It has been suggested that MCP-1 is a contributor to tumor angiogenesis.[6]

Neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2, is a 25 kDa extracellular protein that binds to a range of small hydrophobic molecules and soluble macromolecules.[7] NGAL production is induced by the activation of Toll-like receptors in response to invading bacteria, and it functions to prevent iron sequestration by the pathogen.[8] It is induced in epithelial cells, renal tubular cells, and hepatocytes upon inflammation or injury.[9] High concentrations of NGAL in serum can indicate an inflammatory response.[9]

Tissue inhibitor of metalloproteinases I (TIMP-1) is an endogenous inhibitor of matrix metalloproteinases (MMPs).[10] TIMPs have been implicated in direct regulation of cell growth and apoptosis.[11] TIMP-1 plays a role in pathologic processes associated with rheumatoid arthritis[12] and cardiovascular diseases.[13] TIMP-1 can bind to both activated and latent forms of MMP-9.[14]

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 Inflammation Panel 3 (cyno) Kit are multiplex 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 a SECTOR® Imager 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.

1. MCP-1
2. NGAL
3. TIMP-1
4. *BSA Blocked*

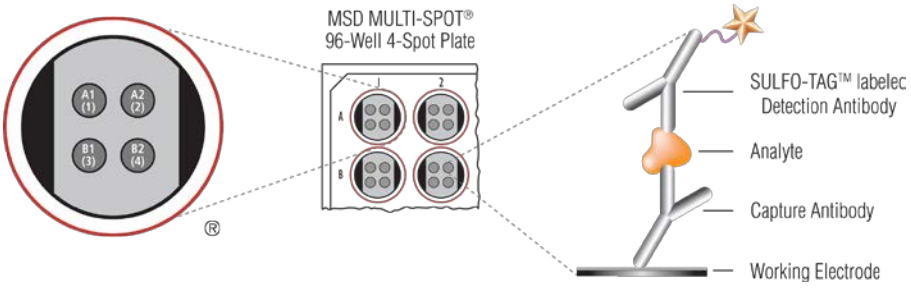


Figure 1. *Spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing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

Reagents Supplied

Product Description	Storage	Quantity per Kit K15191D-1	K15191D-2	K15191D-4
MULTI-SPOT 96-Well 4-Spot Inflammation Panel 3 (cyno) Plate N45191B-1	2–8°C	1 plate	5 plates	25 plates
SULFO-TAG Anti-hu MCP-1 Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-hu NGAL Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-hu TIMP-1 Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
Inflammation Panel 3 (cyno) Calibrator Blend (20X)	≤-70°C	1 vial (20 µL)	5 vials (20 µL ea)	25 vials (20 µL ea)
Diluent 8 R54BA-3 (50 mL)	≤-10°C	1 bottle (50 mL)	4 bottles (50 mL ea)	20 bottles (50 mL ea)
Diluent 11 R55BA-4 (5 mL)	≤-10°C	1 bottle (5 mL)	5 bottles (5 mL ea)	25 bottles (5 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.

Optional Material

Inflammation Control Pak 1 (available for separate purchase from MSD, catalog # C4191-1)

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 8 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 Inflammation Panel 3 (cyno) Kit at 20-fold higher concentration than the recommended highest standard. We recommend a 7-point standard curve with 3-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 by entering K15191D in the search box.

To prepare 7 standard solutions plus a zero calibrator blank for up to 3 replicates:

1) Prepare the highest standard by adding 15 µL of stock calibrator to 285 µL of Diluent 8. Mix well.
2) Prepare the next standard by transferring 100 µL of the highest standard to 200 µL of Diluent 8. Mix well. Repeat 3-fold serial dilutions 5 additional times to generate 7 standards.
3) Use Diluent 8 as the blank.

Dilute Samples

For cynomolgus monkey serum, EDTA plasma, and heparin plasma samples, MSD recommends a 25-fold dilution in Diluent 8; however, you may adjust dilution factors for the sample set under investigation.

To dilute sample 25-fold, add 10 µL of sample to 240 µL of Diluent 8.

Prepare Detection Antibody Solution

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

For 1 plate, combine:

- ❑ 60 µL of 50X SULFO-TAG Anti-hu MCP-1 Antibody
- ❑ 60 µL of 50X SULFO-TAG Anti-hu NGAL Antibody
- ❑ 60 µL of 50X SULFO-TAG Anti-hu TIMP-1 Antibody
- ❑ 2820 µL of Diluent 11

Note: You may omit detection antibody for any analyte not being measured; add 60 µL of Diluent 11 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

Notes

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 30 minutes with vigorous shaking (300–1000 rpm) at room temperature.

Shaking the plate typically accelerates capture at the working electrode.

2. **Wash and Add Sample:** Wash the plate 3 times with 300 µL/well of PBS-T. Add 50 µL of sample (standards, controls, or unknowns) 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.

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

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.

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.

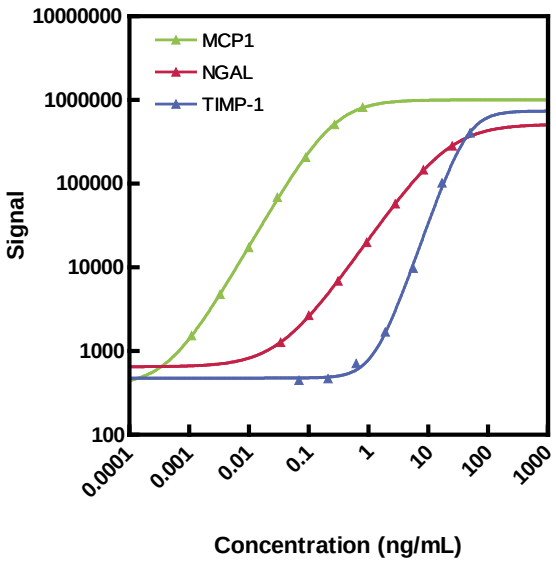
Due to the varying nature of each research application, you should assess assay stability before allowing plates to sit with read buffer for extended periods.

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.



MCP-1		
Conc. (ng/mL)	Average Signal	%CV
0	319	4.1
0.0011	1529	1.5
0.0033	4768	2.5
0.0099	17 328	3.0
0.030	68 671	6.2
0.089	206 871	2.5
0.27	509 259	1.5
0.80	816 754	3.0

NGAL		
Conc. (ng/mL)	Average Signal	%CV
0	489	6.9
0.034	1269	1.7
0.10	2665	5.7
0.31	6867	0.7
0.93	19 939	2.7
2.8	57 496	2.8
8.3	146 601	2.3
25	282 164	3.3

TIMP-1		
Conc. (ng/mL)	Average Signal	%CV
0	340	1.5
0.069	448	5.0
0.21	469	5.7
0.62	710	4.9
1.9	1694	1.2
5.6	9789	3.6
17	102 361	4.5
50	405 350	6.5

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 21 runs.

	MCP-1	NGAL	TIMP-1
Average LLOD (ng/mL)	0.00030	0.015	0.36
LLOD Range (ng/mL)	0.000032–0.00067	0.00067–0.031	0.11–0.79

Precision

Controls were made by spiking calibrator into cynomolgus monkey serum at levels throughout the range of the assay. Analyte levels were measured using a minimum of 2 replicates on 15 runs over 11 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 15 runs.

	Control	Runs	Average Conc. (ng/mL)	Average Intra-run %CV	Inter-run %CV
MCP-1	High	15	0.28	4.6	10.3
	Mid	15	0.028	3.3	5.9
	Low	15	0.0023	3.7	7.3
NGAL	High	15	9.0	3.2	9.2
	Mid	15	1.2	2.9	6.5
	Low	15	0.14	4.1	11.0
TIMP-1	High	15	18	2.4	8.6
	Mid	15	6.8	2.9	8.1
	Low	15	0.91	6.0	11.9

Dilution Linearity

To assess linearity, normal cynomolgus monkey serum samples were diluted 12.5-fold, 25-fold, 50-fold, and 100-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

		MCP-1		NGAL		TIMP-1	
Sample Type	Fold Dilution	Average % Recovery	%Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=10)	25	103	98–116	100	83–124	116	102–129
	50	102	96–111	97	86–110	105	94–119
	100	104	98–114	94	85–100	99	90–106

Spike Recovery

Normal cynomolgus monkey serum, EDTA plasma, and heparin plasma samples were diluted 25-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

	MCP-1			NGAL			TIMP-1		
Sample Type	Spike Conc. (ng/mL)	Average % Recovery	% Recovery Range	Spike Conc. (ng/mL)	Average % Recovery	% Recovery Range	Spike Conc. (ng/mL)	Average % Recovery	% Recovery Range
Serum (N=10)	0.020	109	97–131	0.63	100	93–104	1.3	100	97–105
	0.040	99	91–108	1.3	94	83–106	2.5	101	93–111
	0.080	96	85–103	2.5	99	85–106	5.0	103	92–110
	0.16	97	91–103	5.0	102	85–114	10	103	95–109
	0.32	92	80–100	10	100	86–107	20	98	87–106
EDTA Plasma (N=5)	0.020	113	97–110	0.63	96	90–102	1.3	102	97–110
	0.040	101	102–106	1.3	97	93–103	2.5	102	100–106
	0.080	101	99–104	2.5	103	101–105	5.0	108	104–112
	0.16	98	96–104	5.0	104	100–106	10	112	104–119
	0.32	98	94–103	10	106	102–111	20	113	109–117
Heparin plasma (N=5)	0.020	107	102–113	0.63	103	100–110	1.3	99	88–108
	0.040	104	97–107	1.3	96	94–99	2.5	97	84–111
	0.080	100	99–103	2.5	100	94–106	5.0	100	90–116
	0.16	96	91–103	5.0	98	90–102	10	98	82–115
	0.32	94	87–100	10	97	94–102	20	92	78–100

Specificity

To assess specificity of the detection antibodies, the Inflammation Panel 3 (cyno) was run using blended calibrators with individual detection antibodies (0.27 ng/mL MCP-1; 13 ng/mL NGAL; 33 ng/mL TIMP-1). No signficant cross-reactivity (<0.5%) was observed.

We prepared MMP-2 and MMP-9 at 25, 250, and 2500 ng/mL and tested them with the Inflammation Panel 3 (cyno) to assess interference. No significant interference was observed with either MMP-2 or MMP-9.

Stability

Kit components were tested for freeze-thaw stability. Results (not shown) demonstrated that blended calibrator, controls, and Diluent 8 can go through 3 freeze-thaw cycles without affecting assay performance. Cynomolgus monkey EDTA and heparin plasma samples can go through 3 freeze-thaw cycles without significant changes in their measured concentrations. Do not refreeze cynomolgus monkey serum samples and Diluent 11.

Tested Samples

Normal cynomolgus monkey serum, EDTA plasma and heparin plasma samples were tested at 25-fold dilutions with the Inflammation Panel 3 (cyno). Median and range of concentrations for each sample set are displayed below. Concentrations are corrected for sample dilution.

Sample Type	Statistic	MCP-1	NGAL	TIMP-1
Serum	Median (ng/mL)	0.083	14	347
	Range (ng/mL)	0.027–0.39	6.4–56	213–501
	Number of Samples	20	20	20
	Samples above LLOD	20	20	20
EDTA Plasma	Median (ng/mL)	0.047	7.6	90
	Range (ng/mL)	0.015–0.081	3.2–19	49–264
	Number of Samples	10	10	10
	Samples above LLOD	10	10	10
Heparin Plasma	Median (ng/mL)	0.036	7.2	48
	Range (ng/mL)	0.0092–0.092	1.5–48	<LLOD–202
	Number of Samples	10	10	10
	Samples above LLOD	10	10	9

Assay Components

Calibrators

The assay calibrator blend uses the following recombinant human proteins:

MCP-1 (residues 24-99) expressed in *E. coli*

NGAL (residues 21-198) expressed in murine myeloma cells

TIMP-1 (residues 24-207) expressed in murine myeloma cells

Antibodies

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

References

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Summary Protocol

MSD 96-well MULTI-SPOT Inflammation Panel 3 (cyno) Kit

MSD provides this summary protocol for your convenience. Please read the entire detailed protocol prior to performing the Inflammation Panel 3 (cyno) 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 20-fold in Diluent 8.
- Perform a series of 3-fold dilution steps and prepare a zero calibrator blank.

Dilute samples 25-fold in Diluent 8 before adding to the plate.

Prepare combined detection antibody solution by diluting each stock detection antibody 50-fold in Diluent 11.

Prepare 1X Read Buffer T by diluting 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 30 minutes.

Step 2: Wash and Add Sample

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

Add 50 µL/well of sample (standards, controls, or unknowns).

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.

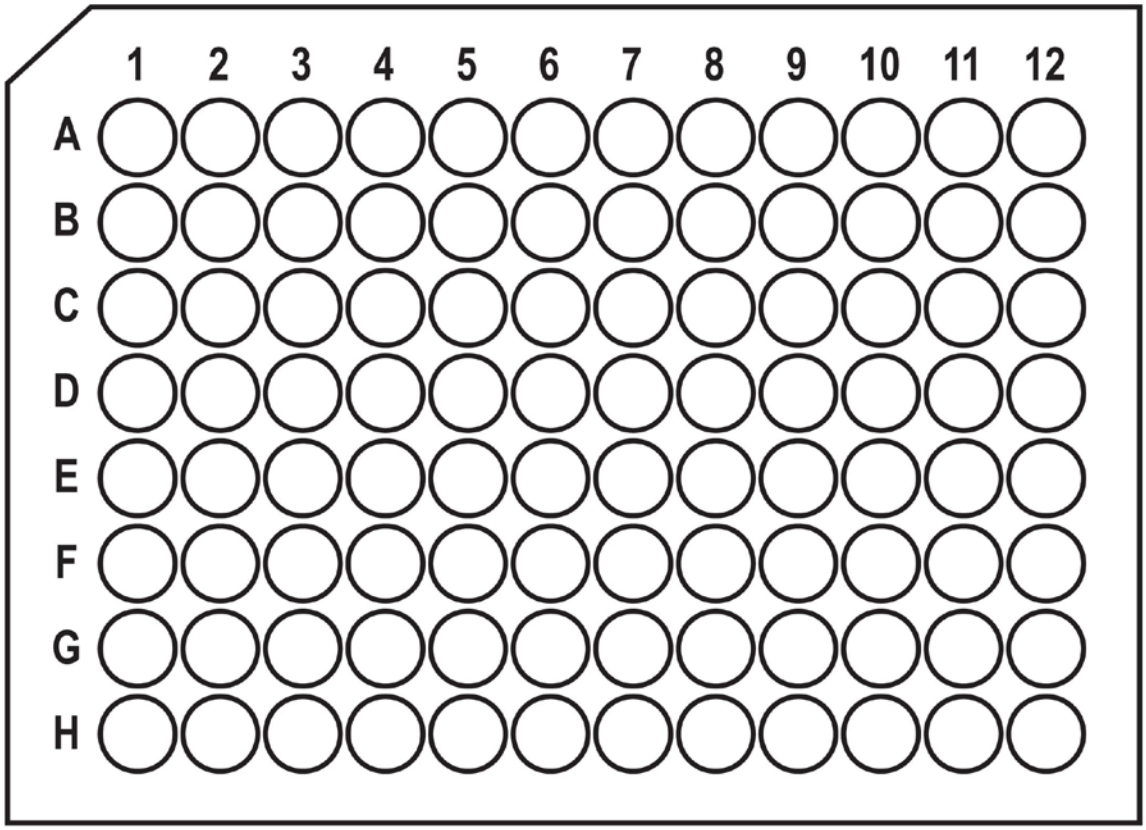
Incubate at room temperature with vigorous shaking (300–1000 rpm) for 2 hours.

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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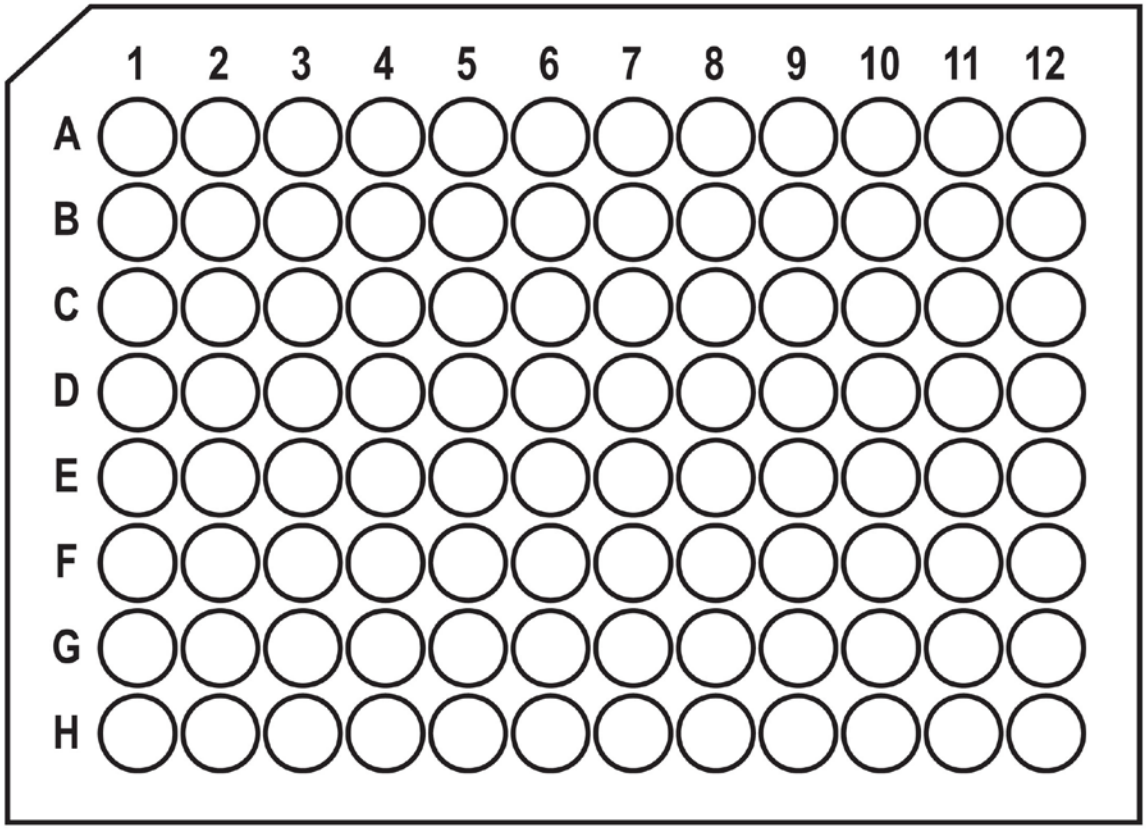
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