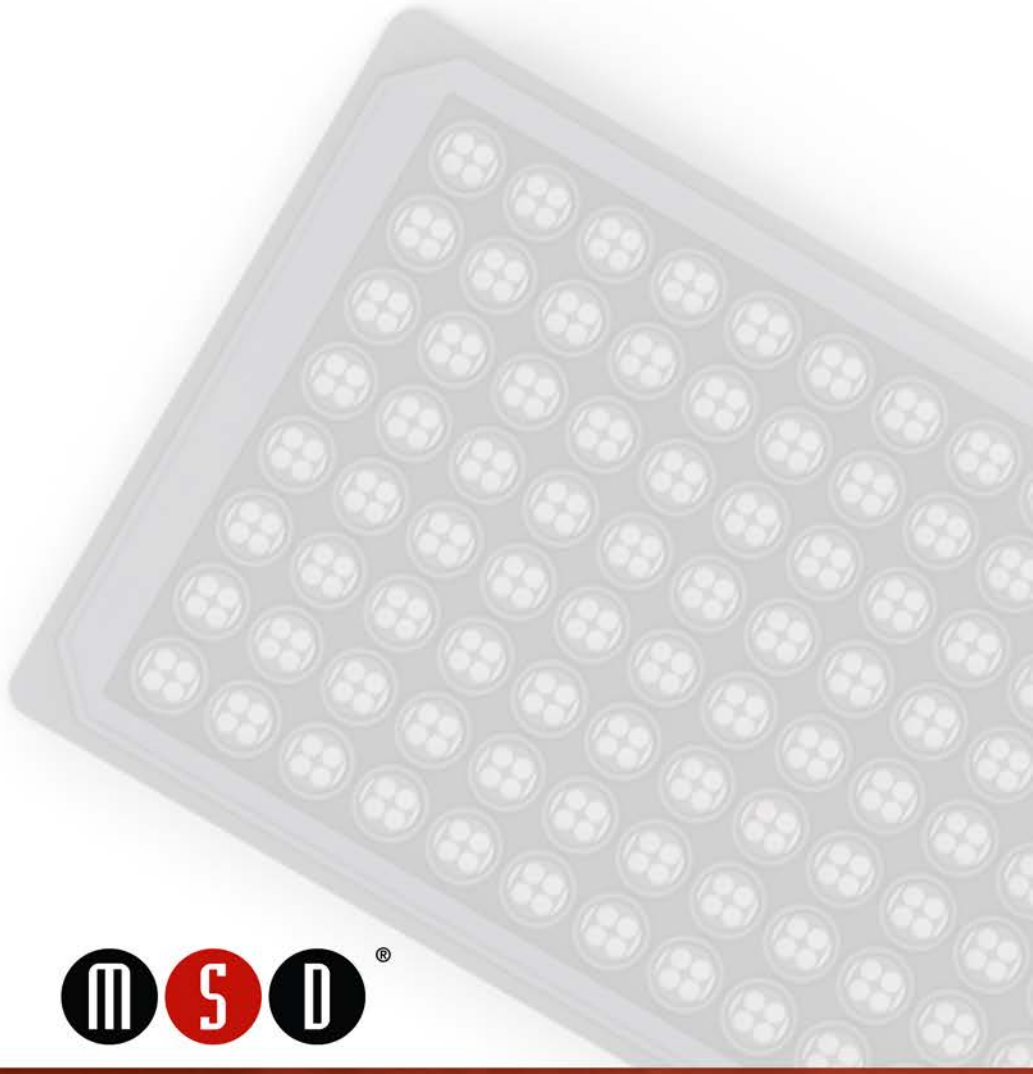
MSD® MULTI-SPOT Assay System

Mouse TH1/TH2 9-Plex Ultra-Sensitive Kit

1-Plate Kit	K15013C-1
5-Plate Kit	K15013C-2
25-Plate Kit	K15013C-4



MSD Biomarker Assays

Mouse TH1/TH2 9-Plex Ultra-Sensitive Kit

IFN-γ, IL-1β, IL-2, IL-4, IL-5, KC/GRO, IL-10, IL-12total, TNF-α

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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MSD Scientific Support

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Introduction

Interferon-γ (IFN-γ), also known as type two interferon, plays a role in the recruitment of leukocytes to the site of infection. IFN-γ is produced by Th1 cells and NK cells. IFN-γ activates macrophages by increasing the expression of major histocompatibility complex (MHC) molecules and antigen processing components. It has also been show to contribute to immunoglobulin (Ig) class switching and suppress Th2 responses. IFN-γ enhances the effects of type one interferons, such as IFN-β.

Interleukin (IL)-1β is produced by dendritic cells, monocytes, macrophages and certain epithelial cells. IL-1β is produced in response to infection induced inflammation. It induces the production of adhesion molecules that enable the transmigration of leukocytes into inflammed tissues. IL-1β also participates in fever induction by the hypothalamus.

IL-2 is produced primarily by activated $CD4^+$ T cells. IL-2 is an important regulator of proliferation and maintenance of several T- and NK-cell subsets. The presence of IL-2 has also been demonstrated to play an important role in the long-term survival of activated helper T cells (Th) and $CD8^+$ cytotoxic T cells.

IL-4 is produced by activated T lymphocytes causing in turn the stimulation and differentiation of B cells and T cell proliferation. IL-4 acts at various stages of cell differentiation and plays an important role in IgE production. It also induces differentiation of uncommitted naïve or Th0-like T cells to Th2 cells.

IL-5 is produced by T helper-2 cells and mast cells. IL-5 is the key cytokine in eosinophil production, activation and localization. IL-5 is associated with asthma and several related allergic disorders.

KC/GRO (keratinocyte chemoattractant; keratinocyte-derieved chemokine/growth related oncogene) also known as CXCL1, GROα, GRO1, NAP-3, and CINC (rat) is a small cytokine belonging to the C-X-C family of chemokines. KC/GRO is produced by macrophages, neutrophils and epithelial cells, and is involved in neutrophil chemoattractant activity. It plays a role in spinal cord development, angiogenesis, tumorigenesis and inflammation.

IL-10 inhibits the production of proinflammatory cytokines by T cells, and it is a potent suppressor of monocyte and macrophage functions. As such, it plays an important role in the regulation and termination of inflammatory responses. IL-10 also plays an important role in the growth and differentiation of B cells, NK cells, Th cells, and cytotoxic T cells. IL-10 is produced by macrophages and certain T cell subsets, including CD4+CD25+Foxp3+ regulatory T cells.

IL-12 participates in the differentiation of naïve T cells in Th1 cells. It stimulates the secretion of IFN-γ and TNF-α and inhibits IL-4 induced proliferation of lymphocytes. IL-12 plays an important role in the mediation of the cytotoxic activity of NK cells and $CD8^+$ cytotoxic T cells. It is produced by dendritic cells, monocytes, macrophages, and B-cells in response to intra-cellular pathogens.

Tumor Necrosis Factor-α (TNF-α) plays a key role in the acute phase reaction and systemic inflammation. TNF-α is primarily produced by activated macrophages, but it is also secreted by a variety of other cell types under pathogenic conditions. Upon receptor binding, it has been shown to trigger diverse cell signaling pathways including apoptosis, proliferation,

differentiation, chemoattraction, hypothalamic regulation, and cytokine production. TNF-α can also contribute to tumorigenesis and viral replication.

Principle of the Assay

MSD assays provide a rapid and convenient method for measuring the levels of protein targets within a single small-volume sample. The assays are available in both singleplex and multiplex formats. In a singleplex assay, an antibody for a specific protein target is coated on one electrode (or "spot") per well. In a multiplex assay, an array of capture antibodies against different targets is patterned on distinct spots in the same well. The Mouse TH1/TH2 9-Plex Assay detects IFN-γ, IL-1β, IL-2, IL-4, IL-5, KC/GRO, IL-10, IL-12total, and TNF-α in a sandwich immunoassay format (Figure 1). MSD provides a plate that has been pre-coated with capture antibody on spatially distinct spots – antibodies for IFN-γ, IL-1β, IL-2, IL-4, IL-5, KC/GRO, IL-10, IL-12total, and TNF-α. The user adds the sample and a solution containing the labeled detection antibodies— anti-IFN-γ, anti-IL-1β, anti-IL-2, anti-IL-4, anti-IL-5, anti-KC/GRO, anti-IL-10, anti-IL-12total, and anti-TNF-α labeled with an electrochemiluminescent compound, MSD SULFO-TAG™ label—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 labeled detection antibodies by bound analytes completes the sandwich. The user adds an MSD read buffer that provides the appropriate chemical environment for electrochemiluminescence and loads the plate into an MSD SECTOR® instrument for analysis. Inside the SECTOR instrument, a voltage applied to the plate electrodes causes the labels bound to the electrode surface to emit light. The instrument measures intensity of emitted light to afford a quantitative measure of IFN-γ, IL-1β, IL-2, IL-4, IL-5, KC/GRO, IL-10, IL-12total, and TNF-α present in the sample.

1 = IFN-γ
2 = IL-1β
3 = IL-2
4 = IL-4
5 = IL-5
6 = KC/GRO
7 = IL-10
8 = IL-12total
9 = *BSA blocked*
10 = TNF-α

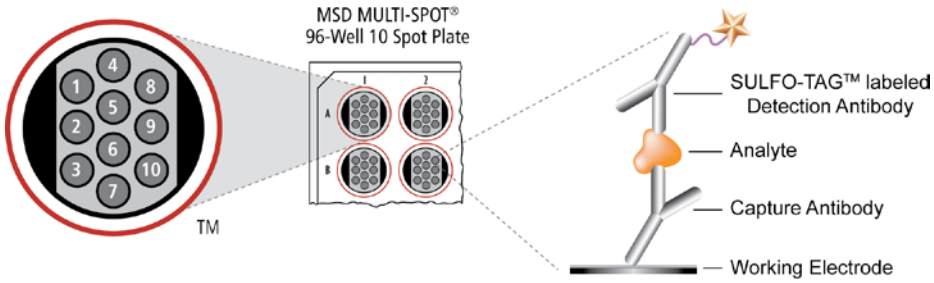


Figure 1. *Spot diagram showing placement of analyte capture antibody. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing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

Reagents Supplied

Product Description	Storage	Quantity per Kit K15013C-1	K15013C-2	K15013C-4
MULTI-SPOT® 96-well 10-Spot Mouse TH1/TH2 9-Plex Plate N05013B-1	2–8°C	1 plate	5 plates	25 plates
SULFO-TAG Detection Antibody Blend[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
Mouse TH1/TH2 9-Plex Calibrator Blend (1 µg/mL of each)	≤-70°C	1 vial (15 µL)	5 vials (15 µL ea)	25 vials (15 µL ea)
Diluent 4 R52BB-4 (8 mL) R52BB-3 (40 mL)	≤-10°C	1 bottle (8 mL)	1 bottle (40 mL)	5 bottles (40 mL 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)
Read Buffer T (4X) R92TC-3 (50 mL) R92TC-2 (200 mL)	RT	1 bottle (50 mL)	1 bottle (50 mL)	2 bottles (200 mL ea)

Required Materials and Equipment - not supplied

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

Safety

Safe laboratory practices and personal protective equipment such as gloves, safety glasses, and lab coats should be used at all times during the handling of all kit components. All hazardous samples should be handled and disposed of properly, in accordance with local, state, and federal guidelines.

[1] SULFO-TAG conjugated detection antibodies should be stored in the dark.

Reagent Preparation

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

> **Important:** Upon first thaw, separate Diluent 4 and Diluent 5 into aliquots appropriate to the size of your assay needs. These diluents can go through up to three freeze-thaw cycles without significantly affecting the performance of the assay.

Prepare Calibrator and Control Solutions

MSD recommends the preparation of an 8-point standard curve consisting of at least 2 replicates of each point. Each well requires 25 µL of Calibrator. For the assay, MSD recommends 4-fold serial dilution steps and Diluent 4 alone for the 8th point:

Standard	Mouse TH1/TH2 9-Plex Calibrator Blend (pg/mL)	Dilution Factor
100X Stock	1000000	
STD-01	10000	100
STD-02	2500	4
STD-03	625	4
STD-04	156	4
STD-05	39	4
STD-06	9.8	4
STD-07	2.4	4
STD-08	0	n/a

To prepare this 8-point standard curve for up to 4 replicates:

1) Prepare the highest Calibrator point (STD-01) by transferring 10 µL of the Mouse TH1/TH2 9-Plex Calibrator Blend to 990 µL Diluent 4.
2) Prepare the next Calibrator by transferring 50 µL of the Mouse TH1/TH2 diluted Calibrator to 150 µL Diluent 4. Repeat 4-fold serial dilutions 6 additional times to generate 7 Calibrators.
3) The recommended 8th Standard is Diluent 4 (i.e. zero Calibrator).

Notes:

a. Alternatively, Calibrators can be prepared in the sample matrix or diluent of choice to verify acceptable performance in these matrices. In general, the presence of some protein (for example, 1% BSA) in the sample matrix is helpful for preventing loss of analyte by adsorption onto the sides of tubes, pipette tips, and other surfaces. If your sample matrix is serum-free tissue culture media, then the addition of 10% FBS or 1% BSA is recommended.

b. The standard curve can be modified as necessary to meet specific assay requirements.

Dilution of Samples

Serum and Plasma

All solid material should be removed by centrifugation. Plasma prepared in heparin tubes commonly displays additional clotting following the thawing of the sample. Remove any additional clotted material by centrifugation. Avoid multiple freeze/thaw cycles for serum and plasma samples. Normal serum or plasma samples may not require a dilution prior to being used in the MSD Mouse TH1/TH2 9-Plex Assay. Serum or plasma with high levels of these analytes may require a dilution.

Tissue Culture

Tissue culture supernatant samples may not require dilution prior to being used in the MSD Mouse TH1/TH2 9-Plex Assay. If using serum-free medium, the presence of carrier protein (e.g., 1% BSA) in the solution is helpful to prevent loss of analyte to the labware. Samples from experimental conditions with extremely high levels of cytokines may require a dilution.

Other Matrices

Information on preparing samples in other matrices, including sputum, CSF, and tissue homogenates can be obtained by contacting MSD Scientific Support at 1-301-947-2025 or ScientificSupport@mesoscale.com.

Prepare Detection Antibody Solution

The Detection Antibody Blend is provided at 50X stock solution. The final concentration of the working Detection Antibody Solution should be at 1X. For each plate used, dilute a 60 µL aliquot of the stock Detection Antibody Blend into 2.94 mL of Diluent 5.

Prepare Read Buffer

The Read Buffer should be diluted 2-fold in deionized water to make a final concentration of 2X Read Buffer T. Add 10 mL of 4X Read Buffer T to 10 mL of deionized water for each plate.

Prepare MSD Plate

This plate has been pre-coated with antibody for the analyte shown in Figure 1. The plate can be used as delivered; no additional preparation (e.g., pre-wetting) is required. The plate has also been exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies.

Assay Protocol

1. **Addition of Diluent 4:** Dispense 25 µL of Diluent 4 into each well. Seal the plate with an adhesive plate seal and incubate for 30 min with vigorous shaking (300–1000 rpm) at room temperature.
2. **Addition of the Sample or Calibrator:** Dispense 25 µL of sample or Calibrator into separate wells of the MSD plate. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
3. **Wash and Addition of the Detection Antibody Solution:** Wash the plate 3 times with PBS-T. Dispense 25 µL of the 1X Detection Antibody Solution into each well of the MSD plate. Seal the plate and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
4. **Wash and Read:** Wash the plate 3 times with PBS-T. Add 150 µL of 2X Read Buffer T to each well of the MSD plate. Analyze the plate on the SECTOR Imager. Plates may be read immediately after the addition of Read Buffer.

Notes

Shaking a 96-well MSD plate typically accelerates capture at the working electrode.

Lower sample volumes such as 10 µL are feasible, but it may reduce assay sensitivity. If lower sample volume is utilized, then the volume of the Calibrators / Standard Curve should be adjusted in parallel.

Bubbles in the fluid will interfere with reliable reading of plate. Use reverse pipetting techniques to insure bubbles are not created when dispensing the Read Buffer.

Analysis of Results

The Calibrators should be run in duplicate to generate a standard curve. The standard curve is modeled using least squares fitting algorithms so that signals from samples with known levels of the analyte of interest can be used to calculate the concentration of analyte in the sample. The assays have a wide dynamic range (3–4 logs) which allows accurate quantitation in many samples without the need for dilution. The MSD DISCOVERY WORKBENCH® analysis software utilizes 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 Standard Curve

The following standard curves are an example of the dynamic range of the assay. The actual signals may vary and a standard curve should be run for each set of samples and on each plate for the best quantitation of unknown samples.

IFN-γ		
Conc. (pg/mL)	Average Signal	%CV
0	440	7.2
2.4	1155	3.5
9.8	3191	2.0
39	10229	3.4
156	36080	1.8
625	132898	3.1
2500	537781	2.1
10000	1981351	2.2

IL-1β		
Conc. (pg/mL)	Average Signal	%CV
0	205	7.8
2.4	428	1.6
9.8	1017	3.3
39	3304	3.6
156	11127	7.2
625	38877	6.3
2500	142907	4.1
10000	472224	3.8

IL-2		
Conc. (pg/mL)	Average Signal	%CV
0	410	5.9
2.4	551	3.9
9.8	1014	2.4
39	2280	5.3
156	7725	4.1
625	29390	2.2
2500	146459	3.4
10000	684529	2.9

IL-4		
Conc. (pg/mL)	Average Signal	%CV
0	639	5.6
2.4	1099	2.9
9.8	2406	6.3
39	7406	3.5
156	26646	3.8
625	97141	4.9
2500	323077	5.6
10000	1112411	3.3

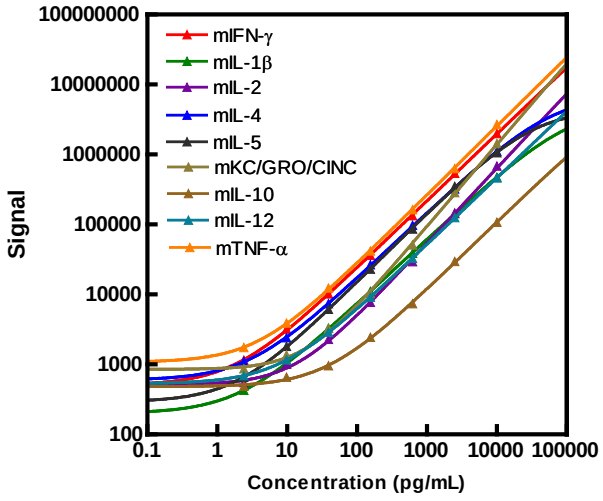
IL-5		
Conc. (pg/mL)	Average Signal	%CV
0	360	8.6
2.4	657	3.2
9.8	1813	2.1
39	6148	4.5
156	23049	2.9
625	85909	2.6
2500	351810	5.7
10000	1061574	3.7

KC/GRO		
Conc. (pg/mL)	Average Signal	%CV
0	583	5.3
2.4	865	2.5
9.8	1363	1.0
39	3267	7.1
156	10363	8.9
625	51204	3.1
2500	285527	9.8
10000	1425208	9.8

IL-10		
Conc. (pg/mL)	Average Signal	%CV
0	465	9.1
2.4	489	7.4
9.8	660	10.1
39	956	8.7
156	2442	2.9
625	7369	3.5
2500	29524	4.5
10000	107699	3.9

IL-12total		
Conc. (pg/mL)	Average Signal	%CV
0	587	9.3
2.4	688	7.4
9.8	1165	7.1
39	2905	4.2
156	9131	5.8
625	32777	6.5
2500	125159	9.6
10000	463889	4.9

TNF-α		
Conc. (pg/mL)	Average Signal	%CV
0	1052	4.9
2.4	1739	2.7
9.8	3916	2.1
39	12343	0.9
156	41706	0.7
625	162874	3.5
2500	629175	3.3
10000	2680895	3.9



Sensitivity

The lower limit of detection (LLOD) is the calculated concentration of the signal that is 2.5 standard deviations over the zero Calibrator. The values below represent the average LLOD over multiple kit lots.

	IFN-γ	IL-1β	IL-2	IL-4	IL-5	KC/GRO	IL-10	IL-12total	TNF-α
LLOD (pg/mL)	0.47	2.1	3.0	0.87	0.70	2.9	11	5.3	1.0

Assay Components

The Mouse IFN-γ, IL-1β, IL-2, IL-4, IL-5, KC/GRO, IL-10, IL-12total, and TNF-α capture and detection antibodies used in this assay are listed below.

	Source species	
Analyte	**MSD Capture Antibody**	**MSD Detection Antibody**
mIFN-γ	Rat Monoclonal	Rat Monoclonal
mIL-1β	Mouse Monoclonal	Goat Polyclonal
mIL-2	Rat Monoclonal	Rat Monoclonal
mIL-4	Rat Monoclonal	Rat Monoclonal
mIL-5	Rat Monoclonal	Rat Monoclonal
mKC/GRO	Rat Monoclonal	Goat Polyclonal
mIL-10	Rat Monoclonal	Goat Polyclonal
mIL-12total	Rat Monoclonal	Goat Polyclonal
mTNF-α	Rat Monoclonal	Rat Monoclonal

Summary Protocol

MSD 96-well MULTI-SPOT Mouse TH1/TH2 9-Plex Ultra-Sensitive Kit

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing the
MSD Mouse TH1/TH2 9-Plex Assay.

Sample and Reagent Preparation

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

If necessary, samples should be diluted in Diluent 4.

Prepare Calibrator solutions and standard curve.

Use the 100X Calibrator stock to prepare an 8-point standard curve by diluting in Diluent 4.

Note: *The standard curve can be modified as necessary to meet specific assay requirements.*

Prepare Detection Antibody Solution by diluting Detection Antibody Blend to 1X in a final volume of 3.0 mL Diluent 5 per plate.

Prepare 20 mL of 2X Read Buffer T by diluting 4X Read Buffer T with deionized water.

SERUM OR PLASMA SAMPLES

Step 1: Add Diluent 4

Dispense 25 µL/well Diluent 4.

Incubate at room temperature with vigorous shaking (300-1000 rpm) for 30 minutes.

Step 2: Add Sample or Calibrator

Dispense 25 µL/well Calibrator or 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 PBS-T.

Dispense 25 µL/well 1X Detection Antibody Solution.

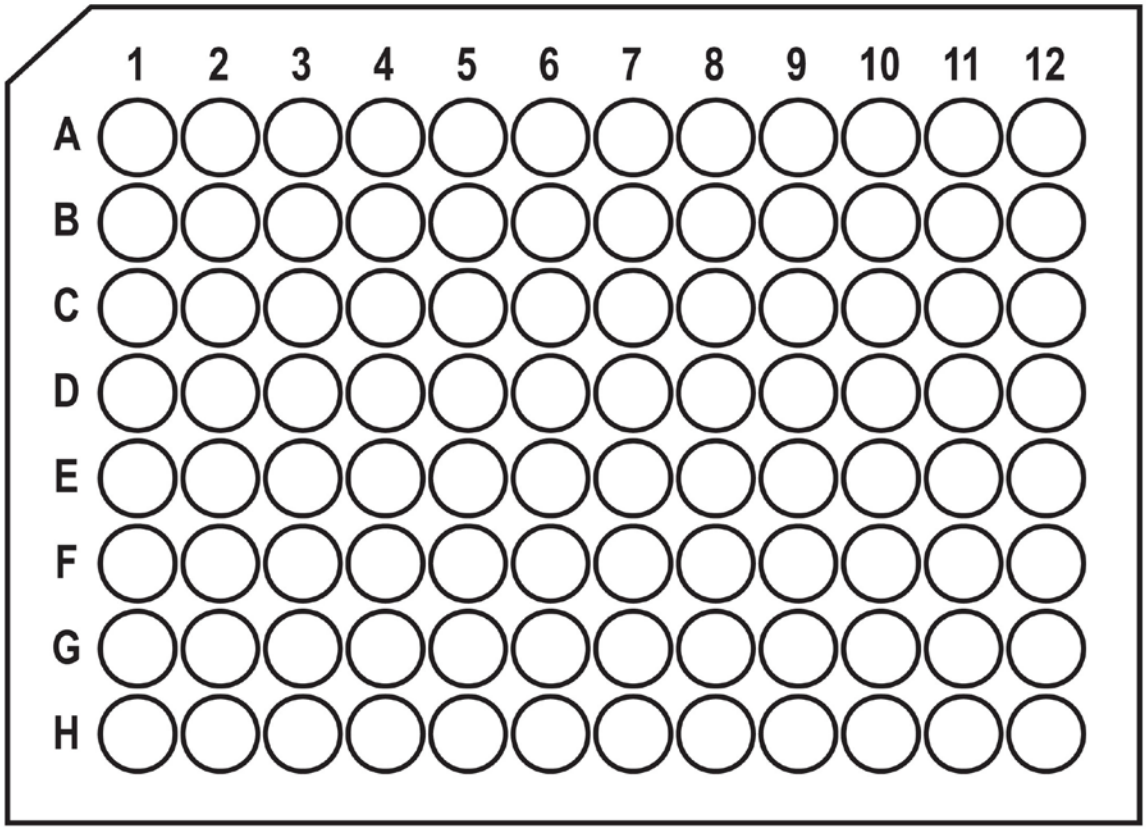
Incubate at room temperature with vigorous shaking (300-1000 rpm) for 2 hours.

Step 4: Wash and Read Plate

Wash plate 3 times with PBS-T.

Dispense 150 µL/well 2X Read Buffer T.

Analyze plate on SECTOR Imager instrument.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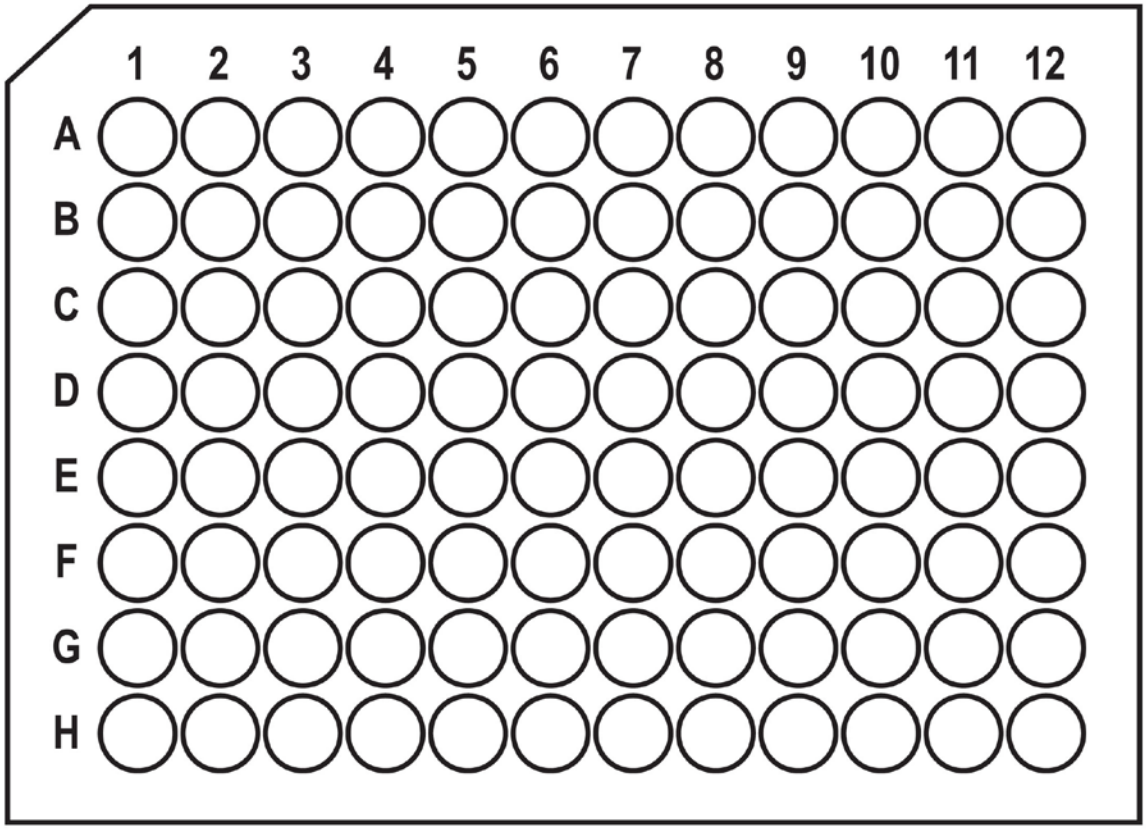
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