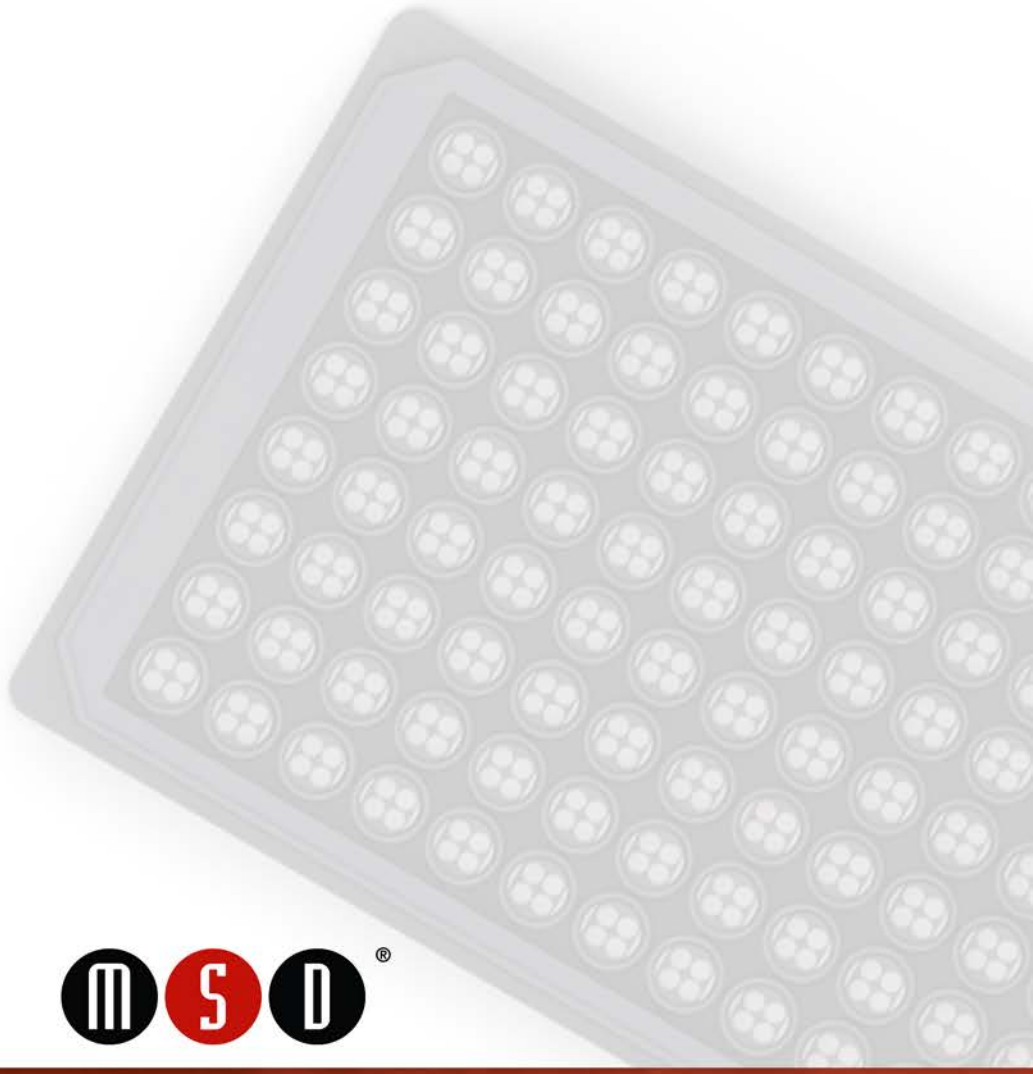
MSD® MULTI-SPOT Assay System

Rat Demonstration 7-Plex Ultra-Sensitive Kit

1-Plate Kit	K15014C-1
5-Plate Kit	K15014C-2
25-Plate Kit	K15014C-4



MSD Biomarker Assays

Rat Demonstration 7-Plex Ultra-Sensitive Kit

IL-1β, KC/GRO, IL-4, IL-5, TNF-α, IFN-γ, IL-13

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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Ordering Information

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Introduction

Inflammatory processes are involved in many physiological events, including infection, the healing response, and other disease states such as autoimmunity. Cytokines and chemokines are small, soluble proteins that can help mediate both acute and chronic inflammatory responses.

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-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.

IL-13 is an important mediator of allergic inflammation and disease and primarily expressed and secreted by T helper type 2 (Th2) cells. Similar to the closely related cytokine IL-4, IL-13 shows multiple effects on functions and differentiation of monocytes and macrophages. In addition to effects on immune cells IL-13 is more importantly implicated as a key player of physiologic changes induced by allergic reactions in diverse tissues.

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.

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 Rat Demonstration 7-Plex Assay detects IL-1β, KC/GRO, IL-4, IL-5, TNF-α, IFN-γ, and IL-13 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 IL-1β, KC/GRO, IL-4, IL-5, TNF-α, IFN-γ, and IL-13 The user adds the sample and a solution containing the labeled detection antibodies— anti-IL-1β, anti-KC/GRO, anti-IL-4, anti-IL-5, anti-TNF-α anti-IFN-γ, and anti-IL-13, 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 IL-1β, KC/GRO, IL-4, IL-5, TNF-α, IFN-γ, and IL-13 present in the sample.

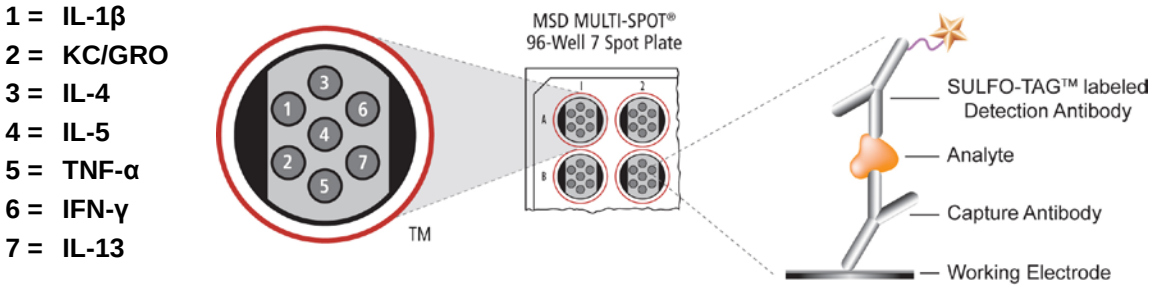


Figure 1. *Spot diagram showing placement of analyte capture antibody. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing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

Reagents Supplied

Product Description	Storage	Quantity per Kit K15014C-1	K15014C-2	K15014C-4
MULTI-SPOT® 96-well 7-Spot Rat Demonstration Panel Plate N75014A-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)
Rat Demonstration 7-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 6 R53BB-4 (8 mL) R53BB-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 6 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

Calibrator for the Rat Demonstration 7-Plex Assay is supplied at 25-fold higher concentration than the recommended highest Calibrator. Prepare the highest Calibrator point (STD-01) by diluting the stock Calibrator 25-fold in Diluent 6. 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 6 alone for the 8th point:

Standard	Rat Demonstration 7-Plex Calibrator Blend (pg/mL)	Dilution Factor
25X Stock	1000000	
STD-01	40000	25
STD-02	10000	4
STD-03	2500	4
STD-04	625	4
STD-05	156	4
STD-06	39	4
STD-07	9.8	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 adding 10 µL of the Rat Demonstration 7-Plex Calibrator Blend to 240 µL Diluent 6.
2) Prepare the next Calibrator by transferring 50 µL of the Rat Demonstration 7-Plex diluted Calibrator to 150 µL Diluent 6. Repeat 4-fold serial dilutions 5 additional times to generate 7 Calibrators.
3) The recommended 8th Standard is Diluent 6 (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 Rat Demonstration 7-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 Rat Demonstration 7-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

Notes

1. **Addition of Diluent 6:** Dispense 25 µL of Diluent 6 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.

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

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.

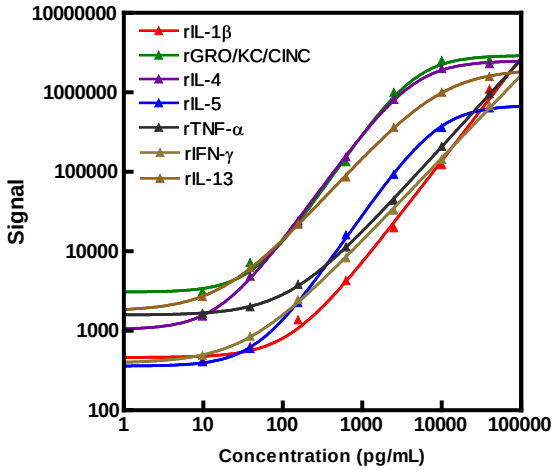
Bubbles in the fluid will interfere with reliable reading of plate. Use reverse pipetting techniques to insure bubbles are not created when dispensing the Read Buffer.

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.



IL-1β		
Conc. (pg/mL)	Average Signal	%CV
0	353	5.8
9.8	421	3.3
39	633	5.8
156	1372	3.5
625	4296	3.1
2500	19784	5.2
10000	123395	6.8
40000	1096809	8.6

KC/GRO		
Conc. (pg/mL)	Average Signal	%CV
0	1802	8.4
9.8	3123	1.3
39	7253	6.9
156	22744	10.8
625	133581	6.5
2500	1004895	3.6
10000	2517396	1.2
40000	2555093	1.0

IL-4		
Conc. (pg/mL)	Average Signal	%CV
0	325	12.2
9.8	1532	5.0
39	4877	3.3
156	24279	5.2
625	155435	5.4
2500	810241	7.0
10000	1994427	7.8
40000	2289231	2.4

IL-5		
Conc. (pg/mL)	Average Signal	%CV
0	382	4.5
9.8	408	2.4
39	596	4.3
156	2258	5.8
625	16088	5.4
2500	92633	7.4
10000	361111	8.3
40000	635474	10.5

TNF-α		
Conc. (pg/mL)	Average Signal	%CV
0	1646	7.6
9.8	1663	2.6
39	2008	3.8
156	3838	2.8
625	11338	2.0
2500	43843	4.7
10000	208698	6.9
40000	960112	8.0

IFN-γ		
Conc. (pg/mL)	Average Signal	%CV
0	356	7.9
9.8	487	4.4
39	854	7.7
156	2471	1.2
625	8286	5.2
2500	33027	6.5
10000	143610	5.3
40000	679143	3.6

IL-13		
Conc. (pg/mL)	Average Signal	%CV
0	1234	5.4
9.8	2694	3.9
39	6255	1.4
156	21866	5.7
625	85934	2.8
2500	363924	7.8
10000	1004996	4.4
40000	1583059	8.6

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.

	IL-1β	KC/GRO	IL-4	IL-5	TNF-α	IFN-γ	IL-13
LLOD (pg/mL)	25	8.3	1.6	25	54	9.0	3.4

Assay Components

The rat IL-1β, KC/GRO, IL-4, IL-5, IFN-γ, TNF-α and IL-13 capture and detection antibodies used in this assay are listed below.

	Source species	
Analyte	**MSD Capture Antibody**	**MSD Detection Antibody**
rIL-1β	Mouse monoclonal	Goat polyclonal
rKC/GRO	Rabbit polyclonal	Goat polyclonal
rIL-4	Mouse monoclonal	Goat polyclonal
rIL-5	Rat monoclonal	Rat monoclonal
rIFN-γ	Mouse monoclonal	Goat polyclonal
rTNF-α	Hamster monoclonal	Goat polyclonal
rIL-13	Goat polyclonal	Goat polyclonal

Summary Protocol

MSD 96-well MULTI-SPOT Rat Demonstration 7-Plex Ultra-Sensitive Kit

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing the
MSD Rat Demonstration 7-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 6.

Prepare Calibrator solutions and standard curve.

Use the 25X Calibrator stock to prepare an 8-point standard curve by diluting in Diluent 6.

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 6

Dispense 25 µL/well Diluent 6.

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.

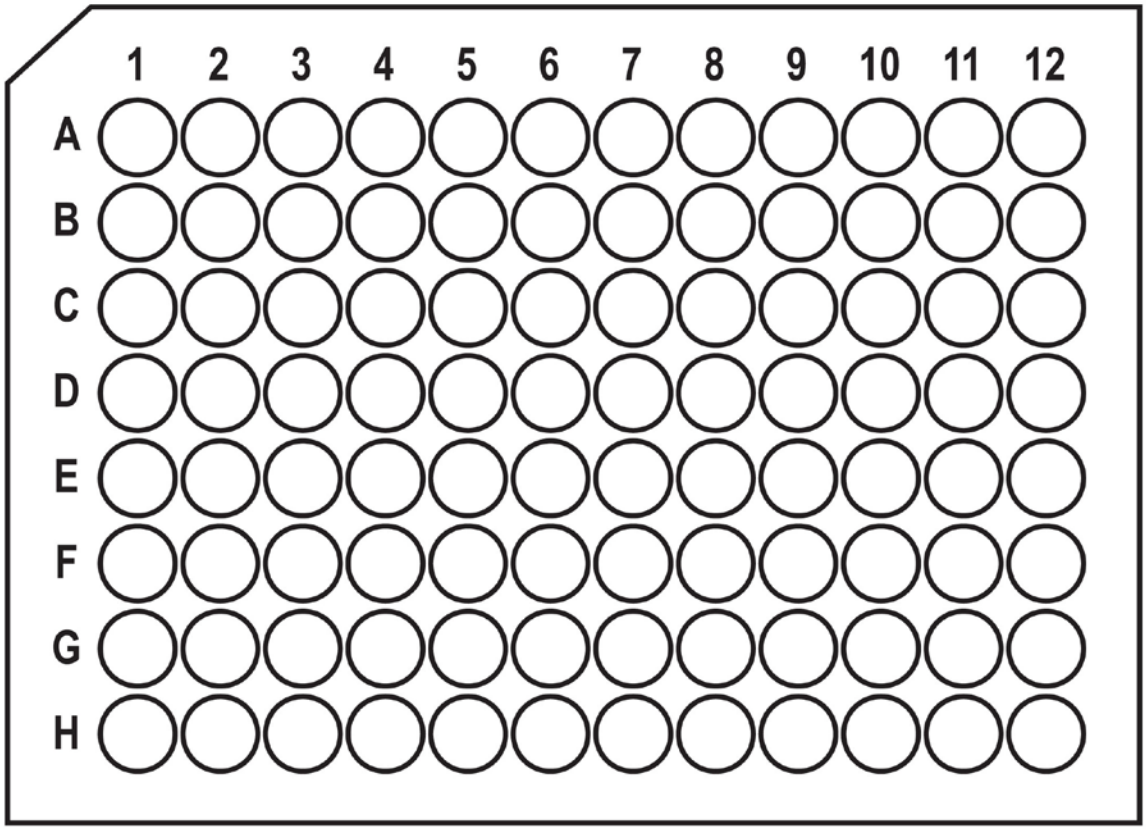
Incubate at room temperature with vigorous shaking (300-1000 rpm) for 2 hours.

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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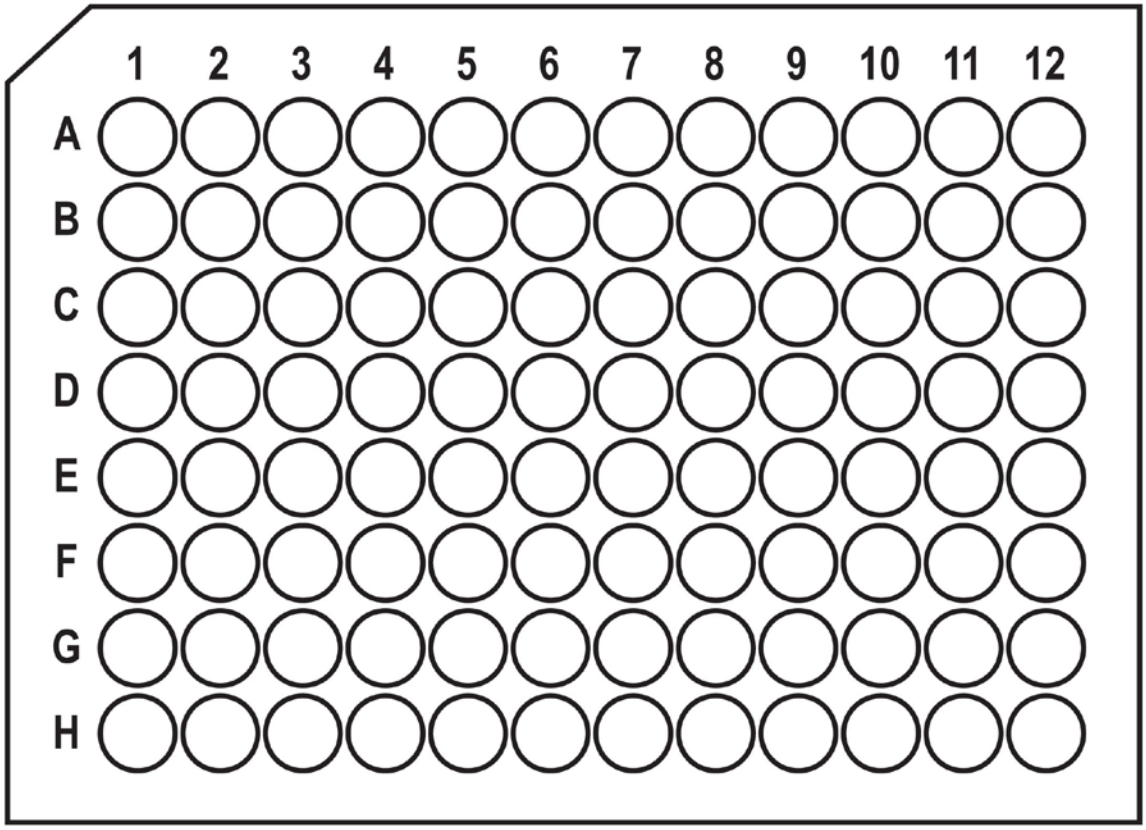
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