MSD® MULTI-SPOT Assay System

Phospho(Thr180/Tyr182)/Total p38 Assay Whole Cell Lysate Kit

1-Plate Kit	K15112D-1
5-Plate Kit	K15112D-2
20-Plate Kit	K15112D-3

www.mesoscale.com®

MSD Cell Signaling Pathway Assays

Phospho(Thr180/Tyr182)/Total p38 Kit

Phospho-p38, Total p38

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®
A division of Meso Scale Diagnostics, LLC.
1601 Research Boulevard
Rockville, MD 20850-3173 USA
www.mesoscale.com

Table of Contents

Ordering Information

MSD Customer Service

Phone: 1-301-947-2085
Fax: 1-301-990-2776
Email: CustomerService@mesoscale.com

MSD Scientific Support

Phone: 1-301-947-2025
Fax: 1-240-632-2219 attn: Scientific Support
Email: ScientificSupport@mesoscale.com

Introduction

The serine/threonine kinase p38 also known as RK, SAPK2A, and CSBP is involved in mediating cellular responses to inflammatory cytokines and environmental stresses such as osmotic shock and UV light.[1] Four isoforms (α, β, γ, δ) of p38 have been identified. Activation of p38 by phosphorylation of threonine 180 and tyrosine 182 is controlled by several upstream kinases including MKK3, MKK6, and MKK4 (SEK).[2] Activated p38 in turn can phosphorylate MAPKAPK2, PRAK kinase, and the transcription factors ATF-2, MAX, CHOP, and MEF2.[3] The p38 signaling pathway regulates various biological processes such as cytokine production, transcriptional regulation, cell proliferation, cell differentiation, and apoptosis.[3]

Principle of the Assay

MSD cell signaling pathway assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. The assays in the Phospho(Thr180/Tyr182)/Total p38 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 MIS 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.

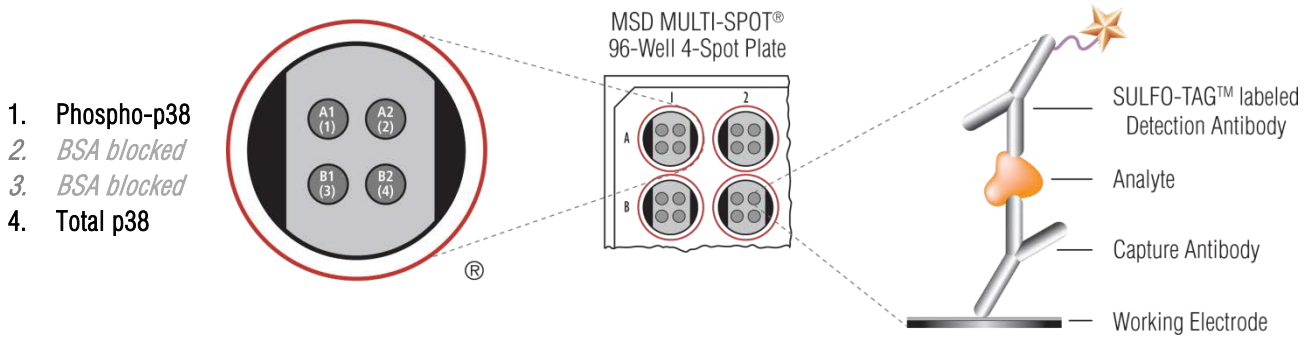

Figure 1. *Spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing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

Reagents Supplied

Product Description	Storage	Quantity per Kit K15112D-1	K15112D-2	K15112D-3
MULTI-SPOT® 96-Well, 4-Spot Phospho(Thr180/Tyr182)/Total p38 Plate N45112A-1	2–8°C	1 plate	5 plates	20 plates
SULFO-TAG Anti-Total p38 Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	4 vials (375 µL ea)
Tris Lysis Buffer (1X) R60TX-3 (50 mL), R60TX-2 (200 mL)	2–8°C	1 bottle (50 mL)	1 bottle (50 mL)	1 bottle (200 mL)
Tris Wash Buffer (10X) R61TX-2 (200 mL), R61TX-1 (1000 mL)	2–8°C	1 bottle (200 mL)	1 bottle (200 mL)	1 bottle (1000 mL)
Phosphatase Inhibitor I (100X)	2–8°C	1 vial (0.1 mL)	1 vial (0.5 mL)	1 vial (2.0 mL)
Phosphatase Inhibitor II (100X)	2–8°C	1 vial (0.1 mL)	1 vial (0.5 mL)	1 vial (2.0 mL)
Protease Inhibitor Solution (100X)	2–8°C	1 vial (0.1 mL)	1 vial (0.5 mL)	1 vial (2.0 mL)
Blocker A (dry powder) R93BA-4	RT	1 vial (15 gm)	1 vial (15 gm)	1 vial (15 gm)
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)

- ❑ Appropriately sized tubes and bottles for reagent preparation
- ❑ Polypropylene microcentrifuge tubes for preparing dilutions
- ❑ 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 (rotary) capable of shaking at 300–1000 rpm
- ❑ Deionized water

Optional Material

MAP Kinase Whole Cell Lysate Set (available for separate purchase from MSD, catalog # C1101-1)

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

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.

Additional safety information is available in the product Material Safety Data Sheet, which can be obtained from MSD Customer Service.

Best Practices and Technical Hints

- Do not mix or substitute reagents from different sources or different kit lots.
- Assay diluent and complete lysis buffer should be kept on ice during the experiment.
- Dilute calibrators, samples, and controls in polypropylene microcentrifuge tubes; use a fresh pipette tip for each dilution; vortex after each dilution before proceeding.
- Avoid prolonged exposure of detection antibody (stock or diluted) to light. During the antibody incubation step, plates do not need to be shielded from light except for direct sunlight.
- Avoid bubbles in wells at all pipetting steps. Bubbles may lead to variable results; bubbles introduced when adding read buffer may interfere with signal detection.
- Use reverse pipetting where necessary to avoid introduction of bubbles, and pipette to the bottom corner of the well when pipetting into empty wells.
- Shaking should be vigorous with a rotary motion between 300 and 1000 rpm.
- When using an automated plate washer, rotating the plate 180 degrees between wash steps may improve assay precision.
- Tap the plate to remove residual fluid after washing.
- Read buffer should be at room temperature when added to the plate.
- Keeping time intervals consistent between adding read buffer and reading the plate should improve inter-plate precision. Limit the time the plate is incubated with read buffer.
- No shaking is necessary after adding read buffer.
- Remove plate seals prior to reading the plate.
- If an incubation step needs to be extended, avoid letting the plate dry out by keeping keep sample or detection antibody solution in the wells.
- When running partial plates, seal the unused portion of the plate with a plate seal to avoid contaminating unused wells. You may adjust volumes proportionally when preparing detection antibody solution.
- After using a partial plate, seal unused sectors with a plate seal. Return plate to its original foil pouch with desiccant pack and seal with tape. Partially used plates may be stored for up to 14 days at 2–8°C.
- If assay results are above the top of the calibration curve, dilute samples and repeat the assay.

Reagent Preparation

Bring all reagents to room temperature.

Prepare Tris Wash Buffer

Dilute the 10X Tris Wash Buffer to 1X as shown below. Tris Wash Buffer (1X) will be used throughout the assay to make additional reagents and wash plates. Approximately 350 mL per plate are required—more if using an automatic plate washer.

For 1 plate, combine:

- ❑ 35 mL Tris Wash Buffer (10X)
- ❑ 315 mL deionized water

Excess Tris Wash Buffer may be stored at room temperature in a tightly sealed container.

Prepare Blocker A Solution

For 1 plate, combine:

- ❑ 600 mg Blocker A (dry powder)
- ❑ 20 mL 1X Tris Wash Buffer

Prepare Antibody Dilution Buffer

For 1 plate, combine:

- ❑ 1 mL Blocker A solution
- ❑ 2 mL 1X Tris Wash Buffer

Set aside on ice.

Prepare Complete Lysis Buffer

Prepare complete lysis buffer just prior to use. The working solution is 1X.

For 1 plate, combine:

- ❑ 50 µL Phosphatase Inhibitor Solution I (100X stock)
- ❑ 50 µL Phosphatase Inhibitor Solution II (100X stock)
- ❑ 50 µL Protease Inhibitor Solution (100X stock)
- ❑ 4.85 mL 1X Tris Lysis Buffer

Immediately place the complete lysis buffer on ice; it should be ice cold before use.

Prepare Detection Antibody Solution

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

For 1 plate, combine:

- ❑ 60 µL 50X SULFO-TAG Anti-Total p38 Antibody
- ❑ 2.94 mL cold antibody dilution buffer

Prepare Read Buffer

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

For 1 plate, combine:

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

You may prepare diluted read buffer in advance and store it at room temperature in a tightly sealed container for up to 1 month.

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.

Sample Preparation

Most lysis buffers and sample matrices are compatible with MSD plates, although high concentrations of denaturing reagents should be avoided. Keep SDS and other ionic detergents to a concentration of 0.1% or less in the sample applied to the well and avoid reducing agents (DTT >1mM). Please contact MSD Scientific Support if you have any questions about lysate preparation options.

Analysis of proteins in their activated state (i.e. phosphorylated) usually requires stimulation prior to cell lysis. Verify cell stimulation and sample preparation prior to using this kit.

Perform all manipulations on ice; keep PBS wash buffer and complete lysis buffer ice cold. Cell concentrations for lysis can range from 0.5 to 5 x 10^7 cells per mL of lysis buffer. Protein yields will vary by cell line. To get your desired final protein concentration, you will need to optimize the number of cells used and the amount of complete lysis buffer added. Depending on the stability of the target in the matrix, you may need additional protease and phosphatase inhibitors in the matrix or diluent.

MSD provides suggested cell lysis protocols in the appendix; however, specific cell types or targets may benefit from alternative buffer components or techniques, depending upon the particular research application.

Protocol

1. **Block Plate:** 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. **Prepare Positive and Negative Cell Lysates:** Thaw cell lysate samples on ice and dilute them immediately before use in ice cold complete lysis buffer. Keep on ice during all manipulations and discard any unused thawed material.
   Lysate samples should be diluted to a working concentration of 6.24–800 µg/mL using complete lysis buffer as prepared above. This will provide 0.156–20 µg of lysate per well.
   You may prepare a dilution series at this point if desired.

3. **Wash and Add Samples:** Wash the plate 3 times with at least 150 µL/well of 1X Tris Wash Buffer. Add 25 µL of sample (standards, controls, or unknowns) per well. Seal the plate with an adhesive plate seal and incubate at room temperature for 3 hours with shaking.

4. **Wash and Add Detection Antibody Solution:** Wash the plate 3 times with at least 150 µL/well of 1X Tris Wash Buffer. Add 25 µL of detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate at room temperature for 1 hour with shaking.

5. **Wash and Read:** Wash the plate 3 times with at least 150 µL/well of Tris Wash Buffer. Add 150 µL of 1X Read Buffer T to each well. Read the plate immediately on the MSD imager.

Typical Data

Representative results for the Phospho(Thr180/Tyr182)/Total p38 Kit using the MSD MAP Kinase Whole Cell Lysate Set (Cat# C1101-1) are illustrated below. The signal and ratio values provided are examples; individual results will vary depending upon the samples tested. Western blot analyses of each lysate type were performed are shown for comparison.

Cell lysate from growing Jurkat cells treated with 50 nM calyculin A and 200 nM PMA for 15 minutes to stimulate phosphorylation (positive) or cell lysate from growing Jurkat treated cells treated with 1 µM rapamycin for 3 hours to inhibit phosphorylation (negative) were added to MSD MULTI-SPOT, 4-spot plates coated with anti-phospho-p38 (Thr180/Tyr182) antibody and anti-total p38 antibody on spatially distinct electrodes in each well. Phospho-p38 (Thr180/Tyr182) and total p38 were detected with anti-total p38 antibody conjugated with MSD SULFO-TAG.

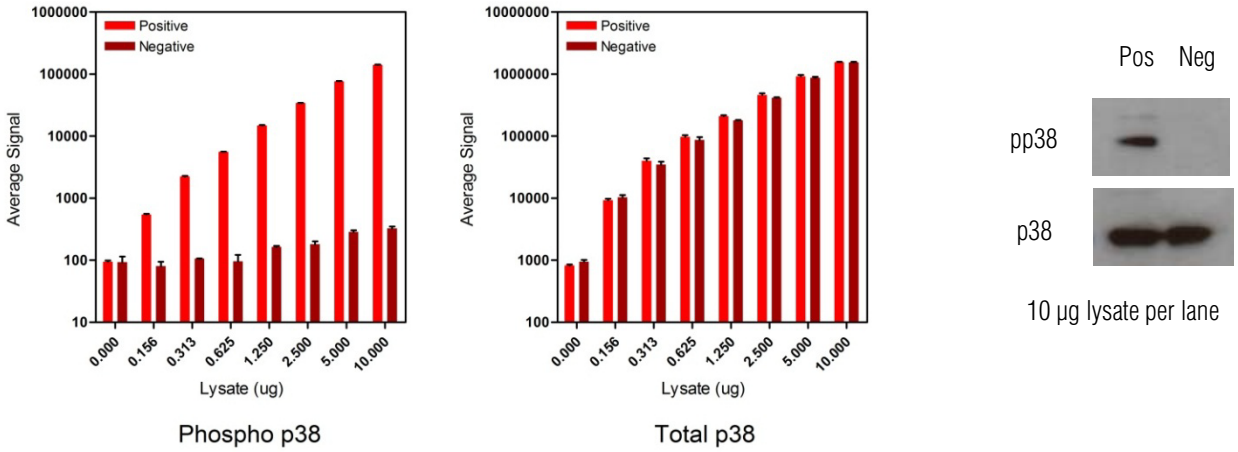

Figure 2: Sample data generated with the Phospho(Thr180/Tyr182)/Total p38 Assay. Increased signal for phospho-p38 (Thr180/Tyr182) was observed with phospho-p38 positive cell lysates, and increased signal for total p38 was observed with both phospho-p38 positive and negative cell lysates. The Phospho(Thr180/Tyr182)/Total p38 Kit provides a measure of the data obtained with the traditional Western blot.

Lysate Titration

Data for positive (P) and negative (N) cell lysates assayed using the Phospho(Thr180/Tyr182)/Total p38 Kit are presented below.

	Lysate (µg)/well	Positive			Negative			P/N
		Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
Phospho-p38	0	95	4	4.5	93	22	23.7	
	0.156	543	18	3.3	80	14	17.7	7
	0.313	2245	47	2.1	106	1	0.7	21
	0.625	5570	68	1.2	96	26	27.4	58
	1.25	14 816	332	2.2	163	7	4.3	91
	2.5	34 217	237	0.7	181	20	10.9	189
	5	76 090	1631	2.1	286	18	6.4	266
	10	140 859	2039	1.4	326	24	7.4	432
Total p38	0	822	34	4.1	946	70	7.4	
	0.156	9302	467	5.0	10 390	849	8.2	0.9
	0.313	39 830	4074	10.2	35 001	3654	10.4	1.1
	0.625	97 571	5892	6.0	86 331	10 517	12.2	1.1
	1.25	208 741	9045	4.3	179 701	2821	1.6	1.2
	2.5	463 331	26 348	5.7	414 084	12 696	3.1	1.1
	5	921 919	49 876	5.4	874 227	33 256	3.8	1.1
	10	1 561 649	18 878	1.2	1 552 999	22 709	1.5	1.0

Assay Components

The capture and detection antibodies used in this assay are listed below. They cross-react with human, non-human primate, mouse, and rat cell lysates.

	Source Species		
Analyte	MSD Capture Antibody	MSD Detection Antibody	Assay Generation
Phospho-p38	Mouse Monoclonal	Mouse Monoclonal	B
Total p38	Mouse Monoclonal	Mouse Monoclonal	B

References

1. Rouse J, et al. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell. 1994 Sep 23;78(6):1027-37.
2. Raingeaud J, et al. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem. 1995 Mar 31;270(13):7420-6.
3. Zarubin T, Han J. Activation and signaling of the p38 MAP kinase pathway. Cell Res. 2005 Jan;15(1):11-8.

Appendix A : Suggested Cell Lysis Protocols

Most lysis buffers are compatible with MSD plates. Cell concentrations for lysis can range from 0.5 to 5 x 10^7 cells per mL of lysis buffer. Protein yields will vary by cell line. To get your desired final protein concentration, you will need to optimize the number of cells used and the amount of complete lysis buffer added. Depending on the stability of the target in the matrix, you may need to add additional protease and phosphatase inhibitors to the matrix or diluent.

MSD provides these suggested cell lysis protocols; however, specific cell types or targets may benefit from alternative buffer components or techniques, depending upon the particular research application.

Preparation in Culture Flask or Petri Dish

Suspension Cells. Pellet cells by centrifugation at 500g for 3 minutes at 2–8°C. Discard supernatant and wash the pellet once with cold PBS. Pellet cells again, discard supernatant, and resuspend in complete lysis buffer at 1–5 x 10^7 cells per mL. Incubate on ice for 30 minutes. (A shorter incubation time of 15 minutes may be adequate for many targets.) Clear cellular debris from the lysate by centrifuging (≥10 000g) for 10 minutes at 2–8°C. Transfer cleared lysate to a fresh tube and discard the pellet. Determine the protein concentration in the lysate using a detergent-compatible protein assay such as a bicinchoninic acid (BCA) assay. Unused lysates should be aliquoted, quickly frozen in a dry ice-ethanol bath, and stored at ≤-70°C.

Adherent Cells. All volumes given are for cells plated on 15 cm dishes. Remove media from the dish and wash cells once with 5 mL cold PBS. Add 2 mL PBS to each dish, scrape the cells from the surface of the dish, and transfer into 15 mL conical tubes. Pellet the cells by centrifugation at 500g for 3 minutes at 2–8°C. Discard supernatant and resuspend cells in 0.5–2 mL of complete lysis buffer per dish. (Alternatively, cells can be lysed by adding 1–2 mL of complete lysis buffer per 15 cm dish after completely removing the PBS wash buffer.) Incubate on ice for 30 minutes.

A shorter incubation time of 15 minutes may be adequate for many targets. Clear cellular debris from the lysate by centrifuging (≥10 000g) for 10 minutes at 2–8°C. Transfer cleared lysate to a fresh tube and discard the pellet. Determine protein concentration in the lysate using a detergent compatible protein assay such as BCA. Unused lysates should be aliquoted, quickly frozen in a dry ice-ethanol bath, and stored at ≤-70°C.

Preparation in 96-well Culture Plate

Successful adaptation to a 96-well culture format depends on cell type and target. First, determine the number of cells of each cell type to be plated per well. MSD generally recommends plating concentrations ranging from 1 x 10^4 to 10^5 cells per well; however, the optimal concentrations will vary depending on cell line used.

Suspension Cells. You may lyse many cell types without removing growth medium. For flat bottom plates, design the experiment so that the final suspension cell volume per well is such that a concentrated complete lysis buffer (prepared by the user) can be added to the well to achieve a final 1X lysis buffer concentration in the well. For example, 40 µL of 5X complete lysis buffer added to a well containing 160 µL of cell culture medium would provide a 1X concentration of complete lysis buffer.

For conical microwell plates, perform lysis by pelleting the cells, removing most of the growth medium, and adding a constant amount of 1X complete lysis buffer.

Adherent Cells. Plate cells on coated tissue culture plates to reduce variability due to cells lost as growth medium is removed. Treat cells as desired. Gently aspirate growth medium from the microwell plate to avoid disrupting the cell monolayer. A PBS wash step is not required and can introduce variability as cells may detach during the wash step. Add 100 µL of 1X complete lysis buffer per well. You may modify lysis volume for different cell types or applications.

You will need to determine the optimum cell lysis time. Some targets are immediately available for detection. Other targets may require an incubation step at room temperature or on ice with gentle agitation.

Carefully pipet cell lysate onto prepared plate and proceed with assay protocol. Note: It is important to transfer a constant volume and to avoid introducing air bubbles by pipetting too vigorously.

Appendix B: Calculating Percent Phosphorylation

The percent phosphoprotein in a sample can be calculated using MSD multiplex assays that include phosphorylated and total protein spots; however, the calculation is not a simple ratio of the phospho-/total signals. Since multiplex assays include the total protein antibody and the phosphorylated protein antibody in the same well, some percentage of the phosphoprotein analytes will be captured by the total protein spot. (The assay's actual "total protein" value combines the signals from both spots.) The formula for percent phosphoprotein includes a relative binding affinity factor (B) in the numerator that adjusts for this.

The percentage of phosphorylated proteins captured by the total protein spot depends on how the binding affinity between the phosphoprotein and its antibody compares to binding affinity between the phosphoprotein and the total protein antibody. This relative binding affinity (B) is easily calculated from the control sample signals (control samples are 100% phosphorylated).

MULTIPLEX ASSAY FORMAT: Anti-total and anti-phospho assays in the same well

% phosphorylation=[(B∗phospho signal)/(phospho signal+total signal)]∗100

Relative Binding Affinity Calculation

B is specific to each assay type and can be calculated using results from the control assay. The formula for B is:

B=(total signal+phospho signal)/phospho signal

Example

Results of control assay:

Phosphoprotein Assay							
Lysates (µg)	Positive Control Lysate			Negative Control Lysate			P/N
	Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
0	245	4	1.4	242	6	2.5	
5.0	19 235	2342	12.2	461	3	0.6	42

Total Protein Assay							
Lysates (µg)	Positive Control Lysate			Negative Control Lysate			P/N
	Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
0	561	18	3.2	569	19	3.4	
5.0	7304	1227	16.8	14530	585	4.0	0.5

B=(total signal+phospho signal)/phospho signal

B=(19 235+7304)/19 235=26 539/19 235=1.3797

B=1.38

Sample calculation of % phosphoprotein for this example:

% phosphorylation=[(B∗phospho signal)/(phospho signal+total signal)] ∗100

% phosphorylation=[(1.38∗19235)/(19235+7304)]∗100=100% (Note: This is the expected answer for controls, which are 100% phosphorylated)

Summary Protocol

Phospho(Thr180/Tyr182)/Total p38 Kit

MSD provides this summary protocol for your convenience. Please read the entire detailed protocol prior to performing the Phospho(Thr180/Tyr182)/Total p38 assay.

Reagent Preparation

Prepare:

- ❑ Wash buffer (dilute 10X Tris Wash Buffer 10-fold in deionized water)
- ❑ Blocker A solution (mix Blocker A powder 3% weight to volume in 1X Tris Wash Buffer)
- ❑ Antibody dilution buffer (dilute Blocker A solution 3-fold in 1X Tris Wash Buffer)
- ❑ Detection antibody solution (dilute 50X detection antibody 50-fold in antibody dilution buffer)
- ❑ Read buffer (dilute 4X Read Buffer T 4-fold with deionized water)

Step 1: Block Plate

Add 150 µL/well of blocking solution.

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

Step 2: Prepare Samples

Prepare complete lysis buffer just prior to sample dilution.

Prepare positive and negative cell lysates and keep on ice until use.

Step 3: Wash and Add Samples

Wash plate 3 times with 150–300 µL/well of Tris Wash Buffer.

Add 25 µL/well of sample (standards, controls, or unknowns).

Incubate at room temperature with vigorous shaking (300–1000 rpm) for 3 hours.

Step 4: Wash and Add Detection Antibody Solution

Wash plate 3 times with 150–300 µL/well of Tris Wash Buffer.

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

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

Step 5: Wash and Read Plate

Wash plate 3 times with 150–300 µL/well of Tris Wash Buffer.

Add 150 µL/well of 1X Read Buffer T.

Analyze plate on SECTOR Imager immediately after adding read buffer.

Plate Diagram