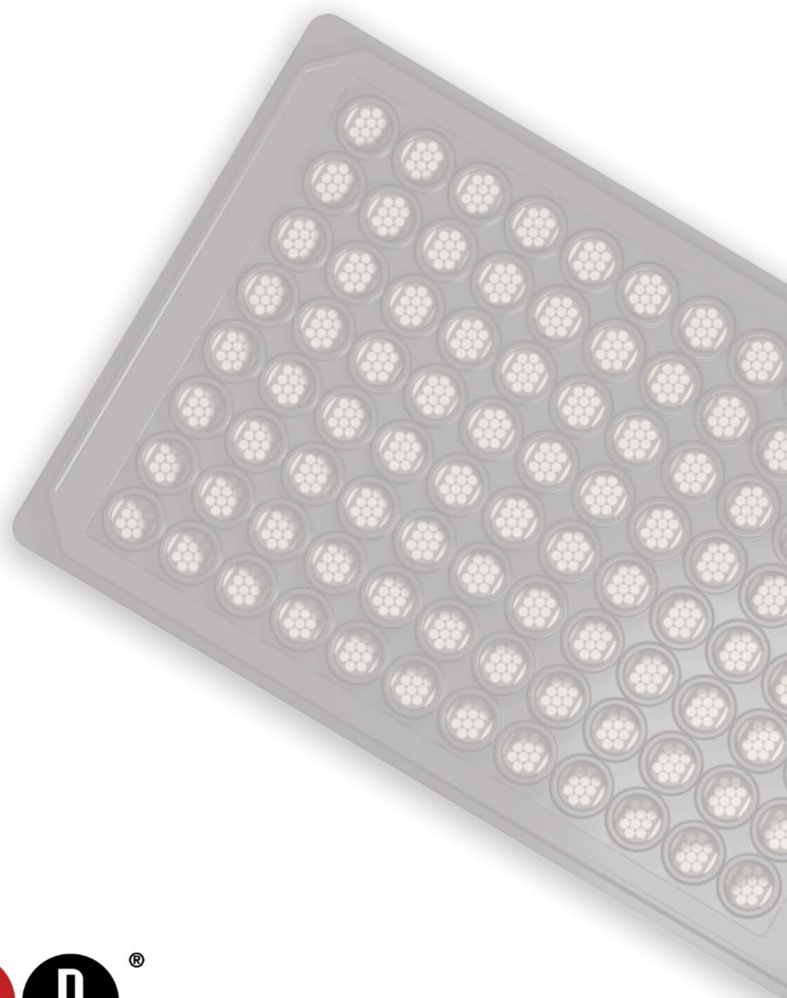
MSD® MULTI-SPOT Assay System

MAP Kinase Panel v2 Kit

1-Plate Kit	K15768U-1
5-Plate Kit	K15768U-2
25-Plate Kit	K15768U-4

T-PLEX®



MSD Cell Signaling Pathway Assays

T-PLEX® MAP Kinase Panel v2 Kit

p38 (phospho)

ERK1/2 (phospho)

JNK (phospho)

For use with human lysates.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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Introduction

This Product Insert describes the T-PLEX MAP Kinase Panel v2 Kit, lists the components, and provides instructions for use.

MAP (Mitogen-Activated Protein) kinases are a family of evolutionarily conserved eukaryotic serine/threonine protein kinases which link receptors on the cell surface to important intracellular regulatory targets. MAP kinases also elicit an intracellular effect in response to physical and chemical cellular stress. MAP kinase cascades within the cell are composed of a series of proteins, the first of which is a MAP kinase kinase kinase (MAPKKK).[1] The MAPKKK is activated by phosphorylation in response to growth factors, mitogens, inflammatory cytokines, G-protein coupled receptors (GPCRs) or stress. The MAPKKK in turn phosphorylates MAPKK, which then phosphorylates MAPK.[2] The activated terminal MAPK translocates into the nucleus, thereby exerting an effect on gene transcription. Through these pathways, the cell regulates responses leading to cell proliferation and differentiation, development, inflammation, and cell survival or apoptosis.[2] ERK1/2, p38, and JNK are all MAP kinases, activated by the MAPK kinases MEK1/2, MKK3/6, and MKK4/7, respectively.[2] Because these pathways are critical for the regulation of cell growth and survival, the MAP kinase family of enzymes offers desirable targets for the development of anti-cancer therapeutics.[3]

Principle of the Assay

MESO SCALE DISCOVERY® (MSD) assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. The assays in the T-PLEX MAP Kinase Panel are sandwich immunoassays (figure 1). MSD provides a plate pre-coated with capture antibodies on independent defined spots in the layout shown below. To run the assay, add 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. Addition of an MSD read buffer creates the appropriate chemical environment for electrochemiluminescence; the plate is loaded into an MSD instrument where a voltage applied to the plate electrodes causes the capture labels to emit light. The instrument measures the intensity of emitted light, which is proportional to the amount of analyte present in the sample, and provides a quantitative measure of each analyte in the sample.

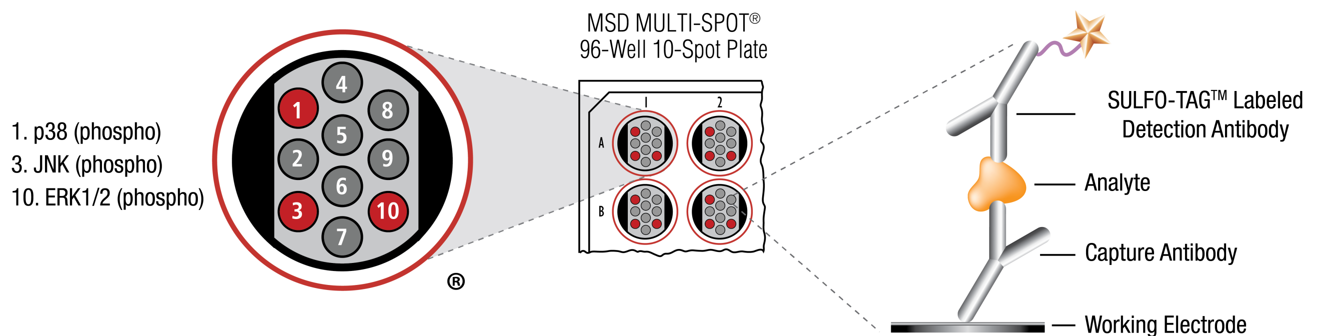

Figure 1. Spot diagram showing placement of analyte capture antibody. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing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

Components

Reagents Supplied With T-PLEX MAP Kinase Panel 1 v2 Kit

Table 1. Components that are supplied with a MAP Kinase Panel 1 v2 Kit.

Component Description	Storage	Quantity per Kit		
		K15768U-1	K15768U-2	K15768U-4
MULTI-SPOT 96-Well 10-Spot MAP Kinase Panel Plate N05768A-1	2–8°C	1 plate	5 plates	25 plates
SULFO-TAG p38 (total) Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG ERK1/2 (total) Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG JNK (phospho) Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 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)	5 bottles (50 mL)
Tris Wash Buffer (10X) R61TX-2 (200 mL), R61TX-1 (1,000 mL)	2–8°C	1 bottle (200 mL)	2 bottles (200 mL)	6 bottles (200 mL)
Phosphatase Inhibitor I (100X)	2–8°C	1 vial (0.1 mL)	1 vial (0.5 mL)	2 vials (2.0 mL)
Phosphatase Inhibitor II (100X)	2–8°C	1 vial (0.1 mL)	1 vial (0.5 mL)	2 vials (2.0 mL)
Protease Inhibitor Solution (100X)	2–8°C	1 vial (0.1 mL)	1 vial (0.5 mL)	2 vials (2.0 mL)
Blocker D-M[2] (2%)	≤-10°C	1 vial (0.2 mL)	1 vial (0.9 mL)	5 vials (0.9 mL ea)
Blocker D-R[2] (10%)	≤-10°C	1 vial (0.05 mL)	1 vial (0.2 mL)	5 vials (0.2 mL ea)
PMSF in 100% DMSO (0.5 M)	≤-10°C	1 vial (0.05 mL)	1 vial (0.25 mL)	5 vials (0.25 mL)
Blocker A (dry powder) R93BA-4	RT	1 vial (15 g)	1 vial (15 g)	1 vial (15 g)
MSD GOLD Read Buffer B R60AM-1 (18 mL), R60AM-2 (90 mL)	RT	1 bottle (18 mL)	1 bottle (90 mL)	5 bottles (90 mL)

Required Materials and Equipment

- Deionized water
- Appropriately sized tubes for reagent preparation
- Polypropylene microcentrifuge tubes for preparing serial dilutions
- Liquid-handling equipment suitable for dispensing 10 to 150 µL into a 96-well microtiter plate
- Plate-washing equipment: automated plate washer or multichannel pipette
- Adhesive plate seals

[1] Some SULFO-TAG conjugated detection antibodies may be light-sensitive, so they should be stored in the dark.
[2] Blockers D-M and D-R can tolerate up to 5 freeze-thaw cycles. Alternatively, an aliquot of Blockers D-M and D-R can be stored at 2-8°C up to 1 month.

- Microtiter plate shaker
- Vortex mixer

Optional Materials

- Phospho-MAPK Whole Cell Lysate Set (C1101-1)

Safety

Use safe laboratory practices. Wear appropriate personal protective equipment, including gloves, safety glasses, and lab coats when handling assay components. Handle and dispose all hazardous samples properly in accordance with local, state, and federal guidelines.

Additional product-specific safety information is available in the applicable safety data sheet(s) (SDS), which can be obtained from MSD Customer Service or at https://www.mesoscale.com/.

Reagent Preparation

Prepare Tris Wash Buffer

Dilute 10X stock of Tris Wash Buffer provided with the MSD kit 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 is required—more if using an automatic plate washer.

For one plate, combine:

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

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

Prepare Blocking Solution

For one plate, combine:

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

Keep Blocking Solution on ice.

Prepare Antibody Dilution Buffer

For one plate, combine:

- ❑ 150 µL 2% Blocker D-M
- ❑ 30 µL 10% Blocker D-R
- ❑ 1 mL blocking solution
- ❑ 1.82 mL 1X Tris Wash Buffer

Set aside on ice.

Prepare Complete Lysis Buffer

To 10 mL of Tris Lysis Buffer provided with the MSD kit, add the following supplemental materials to prepare the complete lysis buffer (sufficient for 2-3 plates):

- ❑ 100 µL Protease Inhibitor Solution (100X stock)
- ❑ 100 µL Phosphatase Inhibitor Solution I (100X stock)
- ❑ 100 µL Phosphatase Inhibitor Solution II (100X stock)
- ❑ 40 µL PMSF in DMSO (250X stock).

Mix well. It is important that PMSF resuspends completely in lysis buffer prior to addition to the cells. To achieve this, mix the solution thoroughly for 5 minutes at room temperature (preferably on a rotator). The complete lysis buffer should be ice cold before use. Use Complete Lysis Buffer as soon as possible following addition of PMSF.

Prepare Detection Antibody Solution

For one plate, combine:

- ❑ 2.82 mL antibody dilution buffer
- ❑ 60 µL 50X SULFO-TAG p38 (total) Antibody (1X final concentration)
- ❑ 60 µL 50X SULFO-TAG ERK1/2 (total) Antibody (1X final concentration)
- ❑ 60 µL 50X SULFO-TAG JNK (phospho) Antibody (1X final concentration)

Read Buffer

MSD GOLD™ Read Buffer B is provided at the working concentration. Do not dilute.

Prepare MSD Plate

This plate has been pre-coated with antibodies for the analytes 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.

Sample Preparation and Storage

This cell lysis protocol is provided as a reference. Specific cell types or targets may benefit from alternative buffer components or techniques, depending upon the particular research application. Most lysis buffers are compatible with MSD MULTI-SPOT® plates, although high concentrations of denaturing detergents (>0.1%) and reducing agents (DTT >1mM) should be avoided. Please contact MSD Scientific Support with any questions regarding lysate preparation options.

All manipulations should be performed on ice. The amount of complete lysis buffer required will vary depending on scale of preparation and type of cells. Larger cells (e.g., NIH3T3, HeLa) should be lysed at concentrations of 1-5 x 10^6 cells per mL of lysis buffer. Smaller cells (e.g., Jurkat) should be lysed at concentrations of 1-5 x 10^7 cells per mL of lysis buffer.

Analysis of proteins in their activated state (i.e., phosphorylated) usually requires stimulation prior to cell lysis. Verification of cell stimulation and sample preparation should be performed prior to using this kit. Phosphate Buffered Saline (PBS) should be ice-cold prior to use.

Suspension Cells

Pellet cells by centrifugation at 500 x g for 3 minutes at 2-8°C. Discard supernatant and wash the pellet once with cold PBS. Pellet the 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 centrifugation greater than or equal to 10,000 x g, at 2-8°C for 10 minutes. Discard the pellet and determine protein concentration in the lysate using a detergent compatible protein assay such as BCA. Unused lysates should be aliquoted and quickly frozen in a dry ice-ethanol bath and stored at ≤-70°C.

Adherent Cells

All volumes are determined for cells plated in 15 cm dishes. Remove media from the plates and wash cells one time with 5 mL cold PBS. Add 2 mL PBS to the plates and scrape the cells from the surface of the dish and transfer into 15 mL conical tubes. Pellet the cells by centrifugation at 500 x g for 3 minutes at 2-8°C. Discard supernatant and resuspend cells in 0.5 – 2 mL of complete lysis buffer per dish. 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 centrifugation greater than or equal to 10,000 x g, at 2-8°C for 10 minutes. Discard the pellet and determine protein concentration in the lysate using a detergent compatible protein assay such as BCA. Unused lysates should be aliquoted and quickly frozen in a dry ice-ethanol bath and stored at ≤-70°C.

Refer to Appendix I for cell lysate preparation protocol modifications that accommodate the use of 96-well culture plates.

Assay Protocol

The following protocol describes the most conservative approach to achieving optimal results. The entire assay, including plate analysis on the MSD reader, can be completed in 5.5 hours. Once desired results are achieved, the protocol can be streamlined to eliminate multiple incubations and wash steps. Samples may be prepared for testing in the manner outlined in the Sample Preparation and Storage section.

1. **Block Plate and Prepare Samples:**
   a. Add 150 µL of blocking solution into each well. Seal the plate with an adhesive plate seal and incubate for 1 hour with vigorous shaking (300–1,000 rpm) at room temperature.
   b. Prepare complete lysis buffer just prior to sample dilution. Keep on ice.
      Note: Samples, including cell lysates, etc., may be used neat or after dilution.
      - MSD plates are compatible with most sample matrices. Avoid reagents that will denature the capture antibodies (e.g., high concentrations of reducing agents such as DTT should be avoided, and also SDS and other ionic detergents should be 0.1% or less in the sample applied to the well).
      - Depending on the stability of the target in the matrix, additional protease and phosphatase inhibitors may be required in the matrix or diluent.
      - If working with purified protein, only a few nanograms per well will generally provide a strong assay signal. Purified recombinant proteins may exhibit differences in both signal and background as compared to native proteins in cell lysates.
      - Keep diluted samples on ice until use
   c. If purchased separately, prepare positive and negative cell lysates:
      - Thaw cell lysate samples on ice and dilute them immediately before use. Keep on ice during all manipulations and discard all remaining thawed, unused material.
      - Dilute cell lysate in complete lysis buffer to a final concentration of 0.8 µg/µL. This will deliver 20 µg/well in 25 µL.
      - A dilution series may also be prepared if desired.

2. **Wash and Add Samples:** Wash the plate 3 times with 450 µL/well of Tris Wash Buffer. Add 25 µL of samples per well. Seal the plate with an adhesive plate seal and incubate for 3 hours with vigorous shaking (300–1,000 rpm) at room temperature.

   Prepare detection antibody solution during this time.

3. **Wash and Add Detection Antibody:** Wash the plate 3 times with 450 µL/well of Tris Wash Buffer. Add 25 µL of detection antibody solution to each well of the MSD plate. Seal the plate with an adhesive plate seal and incubate for 1 hour with vigorous shaking (300–1,000 rpm) at room temperature.

4. **Wash and Read:** Wash the plate 3 times with 450 µL/well of Tris Wash Buffer. Add 150 µL of Read Buffer to each well of the MSD plate.

Assay Components

The capture and detection antibodies used in this assay are listed below.

Table 2. Information on antibodies used in this kit.

	Source Species		
Analyte	MSD Capture Antibody	MSD Detection Antibody	Assay Generation
p38 (phospho)	Mouse Monoclonal	Mouse Monoclonal	C
ERK1/2 (phospho)	Mouse Monoclonal	Mouse Monoclonal	B
JNK (phospho)	Mouse Monoclonal	Rabbit Monoclonal	B

Limitations of the Procedure

The following points should be noted with the T-PLEX MAP Kinase Panel v2 Kit to maximize assay sensitivity and performance.

- A no-wash assay format may be employed, however lower sensitivity may be observed.
- All buffers containing phosphate should be avoided when detecting phosphoproteins.
- Due to the unstable nature of phosphoproteins, cell lysates should be thawed immediately prior to use, and any remaining thawed material should be subsequently discarded.

References

1. Keshet Y, Seger R. The MAP kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. Mol Cancer Ther. Methods Mol Biol. 2010;661:3-38.
2. Zhang YL, Dong C. MAP kinases in immune responses. Cell Mol Immunol. 2005 Feb;2(1):20-7.
3. Dhillon AS, et al. MAP kinase signaling pathways in cancer. Oncogene. 2007 May 14;26(22):3279-90.

Additional Information

Appendix A: Recommended Plate Washer Parameters

Create a program for your automated plate washer with the optimal settings before starting your assay. Example settings for a typical (MSD-recommended) wash program are shown below for a common plate washer (Biotek Model 405 LS).

Table 3. Parameters for customized programs on the Biotek 405 LS microplate washer.

	Wash Program Parameters	Typical Wash Program Settings
	Plate type	96
CYCLES	Wash cycles	3
ASPIRATION	Aspirate Type	TOP
	Travel Rate	1 (4.1% 1.0mm/second)
	Aspirate Delay	0500 milliseconds
	Aspirate X-Position	-35
	Aspirate Y-Position	-35
	Aspirate Height	22
	Secondary Aspirate?	NO
DISPENSE	Dispense Rate	05
	Dispense Volume	0450 µL/well
	Vacuum Delay Volume	0450 µL/well
	Dispense X-Position	00 (0.000 mm)
	Dispense Y-Position	00 (0.000 mm)
	Dispense Height	120 (15.245 mm)
OPTS	Wash Pre dispense?	NO
PRE	Bottom Wash?	NO
MDCYC	Wash Shake?	NO
	Wash Soak?	NO
	Home Carrier?	NO
	Between Cycle Pre-Dispense?	NO
POST	Final Aspirate?	YES
	Aspirate Type	TOP
	Travel Rate	3
	Final Aspirate Delay	0500 milliseconds
	Final Aspirate X-Position	-35 (1.600 mm)
	Final Aspirate Y-Position	-35 (1.600 mm)
	Final Aspirate Height	22
	Secondary Aspirate?	YES
	Final Aspirate Secondary X-Position	35 (1.600 mm)
	Final Aspirate Secondary Y-Position	35 (1.600 mm)
	Final Aspirate Secondary Height	22

96-well Culture Plate Modifications

Successful adaptation to a 96-well culture format is cell type and target-dependent. The number of cells to be plated per well should be determined for each cell type. General recommended plating concentrations for adherent cells range from 1×10^4 – 5×10^4 cells per well and approximately 2×10^6 cells per mL (50 - 75 µL per well) for suspension cells. These numbers are provided as a guide, and the optimal concentrations will vary depending upon cell line used.

Suspension Cells

For flat bottom plates, experiments should be designed such that the final volume per well is 50 – 75 µL. Perform cell lysis using a 4X complete lysis buffer concentrate, supplemented with protease and phosphatase inhibitors at 4X concentrations. Add 4X complete lysis buffer directly to cells in the growth medium for a final 1X concentration in the well.

Note: With some effort, a 10X complete lysis buffer can also be prepared.

(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 biologically treated tissue culture ware (such as BD BioCoat Cellware (Becton, Dickinson and Company, Franklin Lakes, NJ)) to reduce variability due to cells lost as growth medium is removed. Treat cells as desired. Gently aspirate growth medium from microwell plate. A PBS wash step is not required and can introduce variability. Add 50-100 µL 1X complete lysis buffer per well.

Cell lysis time should be determined by the end user. Some targets are immediately available for detection. Other targets may require an incubation step at room temperature, 45°C, or on ice with gentle agitation.

Carefully pipet cell lysate onto prepared capture plate, and proceed with assay protocol.

It is important to transfer a constant volume and avoid pipetting too vigorously, as the introduction of air bubbles may result. (Targets can be captured from a volume greater than 25 µL).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
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F												
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