

MSD[®] MULTI-SPOT Assay System

ERK-STAT3 Cascade Assay Whole Cell Lysate Kit

1-Plate Kit
5-Plate Kit
20-Plate Kit

K15116D-1
K15116D-2
K15116D-3



MSD Phosphoprotein Assays

ERK-STAT3 Cascade

Whole Cell Lysate Kit

Phospho-STAT3 (Tyr705), Phospho-ERK1/2 (Thr202/Tyr204;
Thr185/Tyr187), Phospho-MEK1/2 (Ser217/221)

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.

9238 Gaither Road

Gaithersburg, MD 20877 USA

www.mesoscale.com

MESO SCALE DISCOVERY, MESO SCALE DIAGNOSTICS, WWW.MESOSCALE.COM, MSD, MSD (DESIGN), DISCOVERY WORKBENCH, QUICKPLEX, MULTI-ARRAY, MULTI-SPOT, SULFO-TAG, SECTOR, SECTOR HTS, SECTOR PR, 4-SPOT (DESIGN), and SPOT THE DIFFERENCE are trademarks and/or service marks of Meso Scale Diagnostics, LLC.

BD BioCoat is trademark of Becton Dickinson & Co.

© 2011 Meso Scale Diagnostics, LLC. All rights reserved.

Table of Contents

MSD Advantage.....	4
Introduction	5
Principle of the Assay.....	6
Reagents Supplied	7
Required Material and Equipment – not supplied.....	7
Safety	8
Reagent Preparation.....	8
Sample Preparation and Storage	10
Assay Protocol.....	11
Analysis of Results	13
Typical Data.....	14
Assay Components	18
Limitations of the Procedure	18
References.....	18
Appendix.....	19
Summary Protocol.....	21
Plate Diagrams	23

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

MSD Advantage

MESO SCALE DISCOVERY'S unique spot patterns are a hallmark of our MULTI-ARRAY[®] technology, which enables the measurement of biomarkers utilizing the next generation of electrochemiluminescent detection. In an MSD assay, specific capture antibodies for the analytes are coated in arrays in each well of a 96-well carbon electrode plate surface. The detection system uses patented SULFO-TAG[™] labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of the MULTI-ARRAY and MULTI-SPOT[®] plates. The electrical stimulation is decoupled from the output signal, which is light, to generate assays with minimal background. MSD labels can be conveniently conjugated to biological molecules, are stable, and are non-radioactive. Additionally, only labels near the electrode surface are detected, enabling non-washed assays.

One of the advantages of MSD assays is the minimal sample volume required as compared to a traditional ELISA, which is also limited by its inability to measure more than a single analyte. With an MSD assay, up to ten different biomarkers can be analyzed simultaneously using as little as 10-25 μ L of sample. These assays have high sensitivity, up to five logs of linear dynamic range, and excellent performance in complex biological matrices. Combined, these advantages enable the measurement of native levels of biomarkers in normal and diseased samples without multiple dilutions. Further, the simple and rapid protocols of MSD assays provide a powerful tool to generate reproducible and reliable results. The MSD product line offers a diverse menu of assay kits for profiling biomarkers, cell signaling pathways, and other applications, as well as a variety of plates and reagents for assay development.

Introduction

The **Extracellular Signal-Regulated Kinase 1/2 (ERK1/2)** belongs to the mitogen-activated protein (MAP) kinases family of eukaryotic, serine/threonine protein kinases which link cell surface receptors to important intracellular regulatory targets. The ERK1/2 cascade is a central signaling pathway that regulates more than 100 different substrates and regulates a wide variety of cellular processes including proliferation, differentiation, cell survival, and stress responses.¹ MAP and ERKs are activated by diverse mechanisms including ligation of receptor tyrosine kinases such as epidermal growth factor (EGF) and cell adhesion receptors such as the integrins. Ligand binding of these receptors leads to activation of the small GTPase Ras, which recruits Raf to the membrane, where it is activated. Raf subsequently phosphorylates the dual specificity **MAP/ERK kinase (MEK1/2)** at Ser217 and Ser221 and this activated kinase then phosphorylates ERK at both Thr202 and Tyr204 residues.^{1,2} This event results in full activation of ERK1/2 and leads to its binding of several scaffold or adaptor proteins, such as **Signal Transducer and Activator of Transcription 3 (STAT3)**. This leads to various subcellular and nuclear localization and transactivation of critical regulatory genes.^{1,3}

STAT3 is also activated through phosphorylation (at Tyr705) by Janus kinases (JAKs) and results in rapid translocation to the nucleus where it binds DNA and initiates transcription from specific target genes. STAT3 is of particular interest due to its constitutive phosphorylation in a large proportion of human cancers and its ability to induce neoplastic transformation.^{3,4} This is consistent with STAT3's role in promoting cell cycle progression and cell survival through transactivation of proliferative and apoptotic genes including bcl-2, bcl-xl, mcl-1, and cyclin D1.^{3,4} Intracellular control of several of these signaling cascades is provided by phosphatases which dephosphorylate and inactivate ERK or STAT3, thereby shutting off these pathways. In addition, several feedback mechanisms have also been identified downstream of ERK activation. Hyperactivation of these cell signaling pathways are frequently observed in human malignancies, often as a result of aberrant activation of receptor tyrosine kinases or gain-of-function mutations. Components of the ERK1/2 and STAT pathways are therefore considered attractive candidates for the development of targeted therapies of cancer.¹⁻³

Principle of the Assay

MSD phosphoprotein 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 ERK-STAT3 Cascade Assay uses the sandwich immunoassay format (Figure 1). MSD provides a plate that has been pre-coated with capture antibodies for total STAT3, phospho-ERK1/2 (Thr202/Tyr204; Thr185/Tyr187), and total MEK1/2 on spatially distinct spots. The user adds the sample and a solution containing the detection antibodies—anti-phospho-STAT3 (Tyr705), anti-total ERK1/2, and anti-phospho-MEK1/2 (Ser217/221) conjugated with an electrochemiluminescent compound, MSD SULFO-TAG label—over the course of one or more incubation periods. Analytes in the sample bind to the capture antibodies immobilized on the working electrode surface; recruitment of the conjugated detection antibody by bound analytes complete 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® Imager for analysis. Inside the SECTOR Imager, 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 provide a quantitative measure of phosphorylated STAT3 (Tyr705), ERK1/2 (Thr202/Tyr204; Thr185/Tyr187), and MEK1/2 (Ser217/221) present in the sample.

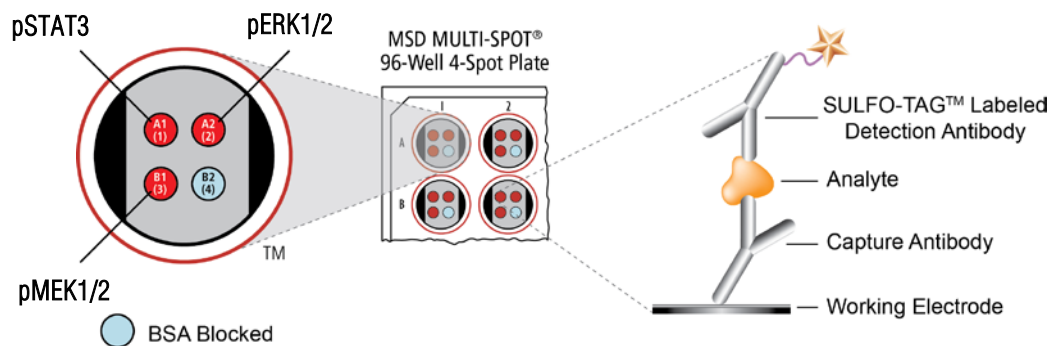


Figure 1. Spot diagram showing placement of analyte capture antibody. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing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

Reagents Supplied

Product Description	Storage	Quantity per Kit		
		K15116D-1	K15116D-2	K15116D-3
MULTI-SPOT 96-Well 4-Spot ERK-STAT3 Cascade Plate N45116B-1	2–8°C	1 plate	5 plates	20 plates
SULFO-TAG Anti-Total ERK1/2 Antibody ¹ (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	4 vials (375 µL ea)
SULFO-TAG Anti-Phospho-MEK1/2 Antibody ¹ (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	4 vials (375 µL ea)
SULFO-TAG Anti-Phospho-STAT3 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 D-M ² (2%)	≤-10°C	1 vial (0.2 mL)	1 vial (0.9 mL)	4 vials (0.9 mL ea)
Blocker D-R ² (10%)	≤-10°C	1 vial (0.05 mL)	1 vial (0.2 mL)	4 vials (0.2 mL ea)
PMSF in 100% DMSO (0.5 M)	≤-10°C	1 vial (0.05 mL)	1 vial (0.25 mL)	1 vial (1.0 mL)
Blocker A (dry powder) R93BA-4	RT	1 vial (15 g)	1 vial (15 g)	1 vial (15 g)
Read Buffer T (4X) R92TC-3 (50 mL), R92TC-2 (200 mL)	RT	1 bottle (50 mL)	1 bottle (50 mL)	1 bottle (200 mL)

Required Materials and Equipment - not supplied

- Deionized water for diluting Tris Wash Buffer (10X) and Read Buffer T (4X)
- 500 mL bottle for reagent preparation
- 50 mL tubes for reagent preparation
- 15 mL tubes for reagent preparation
- Microcentrifuge tubes for preparing serial dilutions

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.

- 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.

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

Prepare Antibody Dilution Buffer

For one plate, combine:

- 150 μ L 2% Blocker D-M
- 30 μ L 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 μ L Protease Inhibitor Solution (100X stock)
- 100 μ L Phosphatase Inhibitor Solution I (100X stock)
- 100 μ L Phosphatase Inhibitor Solution II (100X stock)
- 40 μ L Phenylmethanesulfonylfluoride (PMSF) in Dimethylsulfoxide (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 μ L 50X SULFO-TAG Anti-Total ERK1/2 Antibody (1X final concentration)
- 60 μ L 50X SULFO-TAG Anti-Phospho-MEK1/2 Antibody (1X final concentration)
- 60 μ L 50X SULFO-TAG Anti-Phospho-STAT3 Antibody (1X final concentration)

Prepare Read Buffer

For one plate, combine:

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

Diluted read buffer may be stored at room temperature in a tightly sealed container for later use.

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 $1-5 \times 10^6$ cells per mL of lysis buffer. Smaller cells (e.g. Jurkat) should be lysed at concentrations of $1-5 \times 10^7$ 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 $500 \times g$ for 3 minutes at $2-8^\circ\text{C}$. Discard supernatant and wash the pellet once with cold PBS. Pellet the cells again, discard supernatant and resuspend in complete lysis buffer at $1 - 5 \times 10^7$ 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 $10000 \times g$, at $2-8^\circ\text{C}$ 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 $\leq -70^\circ\text{C}$.

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 $500 \times g$ for 3 minutes at $2-8^\circ\text{C}$. 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 $10000 \times g$, at $2-8^\circ\text{C}$ 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 $\leq -70^\circ\text{C}$.

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 with the MULTI-SPOT ERK-STAT3 Cascade Assay. 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 (300–1000 rpm) at room temperature.
- b. Prepare complete lysis buffer just prior to sample dilution.

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. Prepare positive and negative cell lysates:
(if purchased separately).
 - 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 $\mu\text{g}/\mu\text{L}$. This will deliver 20 μg /well lysate in 25 μL .
 - A dilution series may also be prepared if desired.

Notes

Read entire protocol prior to beginning the assay.

Solutions containing MSD Blocker A should be stored at 2-8°C and discarded after 14 days.

Complete lysis buffer should be kept ice-cold during all experimental manipulations.

The sensitivity of MSD immunoassays rivals that of ELISAs and Western blots. The amount of sample required for a given assay will depend on the abundance of the analyte in the matrix and the affinities of the antibodies used.

Samples and standards cannot be serially diluted in the MSD plate. Use microcentrifuge tubes or a separate 96-well polypropylene plate to prepare dilutions.

2. **Wash and Add Samples:** Wash the plate 3 times with 300 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 (300–1000 rpm) at room temperature.

Prepare detection antibody solution during this time.

3. **Wash and Add Detection Antibody:** Wash the plate 3 times with 300 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 (300–1000 rpm) at room temperature.

Prepare 1X Read Buffer T during this time.

4. **Wash and Read:** Wash the plate 3 times with 300 µL/well of Tris Wash Buffer. Add 150 µL of 1X Read Buffer T to each well of the MSD plate.

Analyze the plate on the SECTOR Imager:

- a. Double click on DISCOVERY WORKBENCH[®] icon on computer desktop (if not already open).
- b. Click the SECTOR Imager icon in upper left corner of screen (if not already open to plate reading screen).
- c. From the pull down menu select “Read From Barcode.”
- d. If only reading one plate check “Return Plate to Input Stack.” Then check “Read Plates(s)” checkbox and enter 1.
- e. If reading multiple plates, check the “Read Plate(s)” checkbox and enter number of plates to be read in the text field. For example, if five plates need to be read, type in “5.”
- f. Click the “Run” button. The “Run Options” window will be displayed
- g. If the data from each microplate is to be exported as individual files, select “Separate Files” in the “Export” area of the “Run Options” window. Select “Appended File” if all data from the entire stack run is to be exported to one file. Select “Default” in the “Export Format” area. Check the box to export default data file.
- h. If desired, make selections to export a custom data file.
- i. Browse and select the location to export data files.
- j. Click OK to initiate the run.
- k. Data will be automatically saved in the software database. Text versions of the requested data files will be exported to the designated folder.

Notes

Shaking a 96-well MSD MULTI-ARRAY or MULTI-SPOT plate during an incubation step will typically accelerate capture at the working electrode.

The lysate sample incubation time provided is optimized for the use of MSD cell lysates. Samples from other sources may require a longer incubation.

Excess diluted read buffer may be kept in a tightly sealed container at room temperature for later use.

Bubbles introduced during the read buffer addition will interfere with imaging of the plate and produce unreliable data.

Plate should be imaged within 5 minutes following the addition of read buffer. Due to the varying nature of each research application, assay stability should be investigated prior to allowing plates to sit with read buffer for extended periods.

An all-inclusive indelible copy of the data and associated instrument information will be saved on the internal database, regardless of data file export selection. Additional copies of the data can be exported in any layout at a later time using this database. Consult the instrument user manual for more information.

Analysis of Results

The percent phosphoprotein in a sample can be calculated using independent MSD phosphoprotein and total protein singleplex assays or MSD phospho-/total multiplex phosphoprotein assays.

INDEPENDENT ASSAY FORMAT: Anti-Total Singleplex and Anti-Phospho-Singleplex Assays

$$\% \text{ Phosphoprotein} = (\text{Phospho-signal} / \text{Total signal}) \times 100$$

MULTIPLEX ASSAY FORMAT: Anti-Total and Anti-Phospho-Assay in the same well

$$\% \text{ Phosphoprotein} = ((2 \times \text{Phospho-signal}) / (\text{Phospho-signal} + \text{Total signal})) \times 100$$

Note:

1. The above calculation assumes that the capture antibodies on the anti-phospho and anti-total spots have very similar binding affinities.
2. The numerator in the equation contains a distribution factor of 2 based on the assumption that the phosphorylated isoform of the protein binds with a similar affinity to the phospho-specific and total capture antibodies. Given equivalent binding of the phosphorylated isoform to both capture antibodies, half of the phosphorylated species will be captured by the phospho-specific and the other half will be captured by the phosphorylation-independent (total) antibody. Therefore, the phospho-specific signal can be referred to as 2X of the phospho spot.
3. The denominator is "phospho + total" because this represents the total of all the analyte captured on both of the spots.
4. If the % phosphorylation is > 100%, then the distribution factor in the numerator may be adjusted to less than 2X such that the % phosphorylation with the control lysates is 100%.

Example:

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	26.0	
5.0	19235	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

$$\% \text{ Phosphoprotein} = [(2 \times \text{Phospho signal}) / (\text{Phospho signal} + \text{Total signal})] \times 100$$

Therefore, % phosphoprotein with 5 μg of positive lysate will be:

$$[(2 \times 19235) / (19235 + 7304)] \times 100 = 144\% \text{ phosphorylation}$$

In this case, the constant in the numerator may be adjusted using the control lysates as follows:

$$[(1.38 \times 19235) / (19235 + 7304)] \times 100 = 100\% \text{ phosphorylation}$$

1.38 should be used as the numerator for further calculations in the same experiment.

Typical Data

Representative results for the MULTI-SPOT ERK-STAT3 Cascade Assay are illustrated below. The signal and ratio values provided below are example data; individual results may vary depending upon the samples tested.

Results with HeLa cells treated with Oncostatin M:

Confluent HeLa cells (negative) were treated with sodium vanadate (1 mM, 4 hours) and oncostatin M (40 ng/mL, 5 minutes) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-phospho-ERK1/2, anti-total MEK1/2, and anti-total STAT3 antibodies on three of the four spatially distinct electrodes per well. Phosphorylated ERK1/2, MEK1/2 and STAT3 were detected with anti-total ERK1/2, anti-phospho-MEK1/2, and anti-phospho-STAT3 antibodies conjugated with MSD SULFO-TAG reagent. Western blot analyses of each lysate type were performed with phospho-ERK1/2, phospho-MEK1/2, and phospho-STAT3 antibodies and are shown below for comparison.

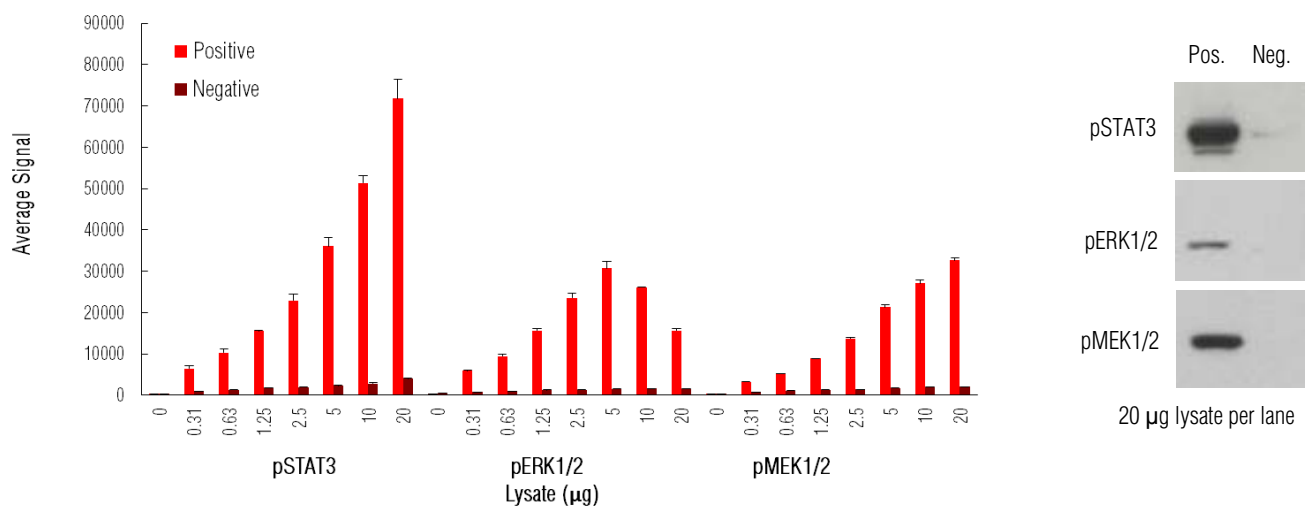


Figure 2: Sample data generated with MULTI-SPOT ERK-STAT3 Cascade Assay. Increased signals were observed with only ERK-STAT3 Cascade positive cell lysate. Signals for negative lysate remained low throughout the titration for all assays. The ERK-STAT3 Cascade Assay provides a quantitative measure of the data obtained with the traditional Western blot.

Lysate Titration

Data for positive and negative HeLa cell lysates using the MULTI-SPOT ERK-STAT3 Cascade Assay are presented below.

	Lysate (µg)	Positive			Negative			P/N
		Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
pSTAT3	0	282	4	1.5	284	6	2.0	
	0.31	6460	557	8.6	937	11	1.1	6.9
	0.63	10314	776	7.5	1262	38	3.0	8.2
	1.3	15509	186	1.2	1664	14	0.8	9.3
	2.5	22944	1607	7.0	1885	18	0.9	12
	5.0	36039	2024	5.6	2279	114	5.0	16
	10	51344	1749	3.4	2824	271	9.6	18
	20	71772	4660	6.5	3941	88	2.2	18
pERK1/2	0	346	26	7.6	382	30	8.0	
	0.31	5847	243	4.2	720	26	3.6	8.1
	0.63	9469	449	4.7	923	76	8.3	10
	1.3	15579	570	3.7	1232	25	2.0	13
	2.5	23598	1155	4.9	1320	18	1.3	18
	5.0	30672	1792	5.8	1500	75	5.0	20
	10	26111	29	0.1	1589	16	1.0	16
	20	15584	537	3.4	1507	15	1.0	10
pMEK1/2	0	324	7	2.2	338	2	0.6	
	0.31	3181	89	2.8	778	18	2.4	4.1
	0.63	5215	20	0.4	1026	38	3.7	5.1
	1.3	8896	8	0.1	1330	13	1.0	6.7
	2.5	13667	318	2.3	1419	18	1.2	9.6
	5.0	21299	492	2.3	1696	1	0.0	13
	10	27110	759	2.8	1978	22	1.1	14
	20	32608	550	1.7	1989	34	1.7	16

Results with Jurkat cells treated with PMA: Control Lysates for Phosphorylated ERK1/2 and MEK1/2

Jurkat cells were treated with LY294002 (50 μ M, 2.5 hours) (negative) or with PMA (200 nM, 15 minutes) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-phospho-ERK1/2, anti-total MEK1/2, and anti-total STAT3 antibodies on three of the four spatially distinct electrodes per well. Phosphorylated ERK1/2, MEK1/2, and STAT3 were detected with anti-total ERK1/2, anti-phospho-MEK1/2, and anti-phospho-STAT3 antibodies conjugated with MSD SULFO-TAG reagent. Western blot analyses of each lysate type were performed with phospho-ERK1/2, phospho-MEK1/2, and phospho-STAT3 antibodies and are shown below for comparison.

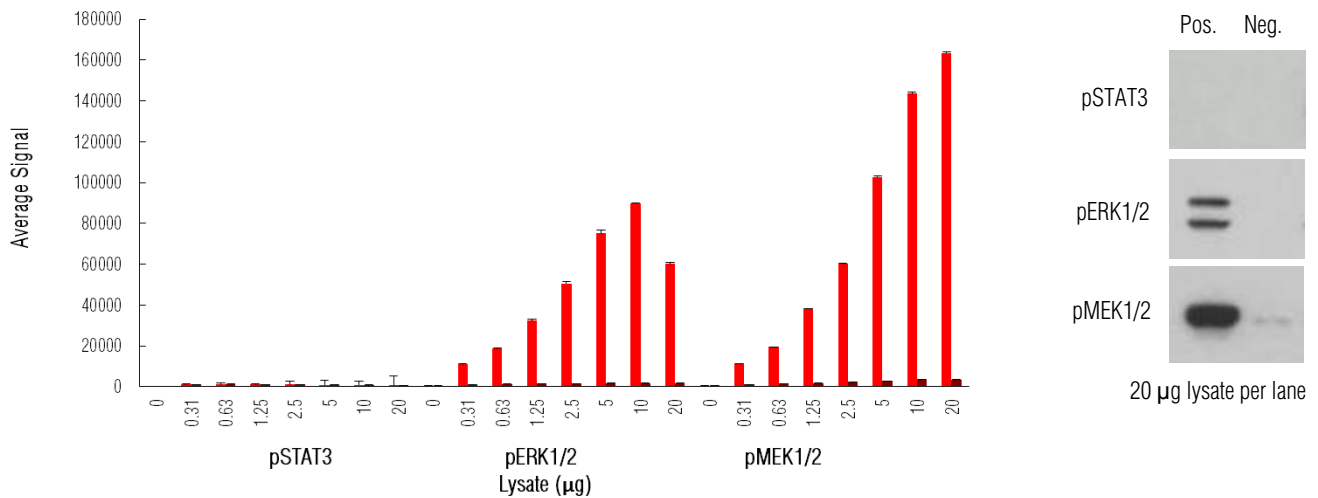


Figure 3: Sample data generated with MULTI-SPOT ERK-STAT3 Cascade Assay. Increased signals were observed for pERK1/2 and pMEK1/2 assays with the titration of ERK-STAT3 Cascade positive cell lysate. Signals for pSTAT3 assay remained low. Signals for negative lysate also remained low throughout the titration for all assays. The ERK-STAT3 Cascade Assay provides a quantitative measure of the data obtained with the traditional Western blot.

Lysate Titration

Data for positive and negative Jurkat cell lysates using the MULTI-SPOT ERK-STAT3 Cascade Assay are presented below.

	Lysate (µg)	Positive			Negative			P/N
		Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
pSTAT3	0	310	11	3.6	314	22	7.0	
	0.31	999	46	4.6	975	0	0.0	1.0
	0.63	1170	62	5.3	1209	23	1.9	1.0
	1.3	1146	10	0.9	1134	23	2.0	1.0
	2.5	1000	13	1.3	974	62	6.3	1.0
	5.0	897	40	4.4	851	15	1.7	1.1
	10	798	25	3.1	755	13	1.7	1.1
	20	735	0	0.0	642	4	0.7	1.1
pERK1/2	0	365	20	5.4	389	17	4.4	
	0.31	10840	472	4.4	904	0	0.0	12
	0.63	18559	854	4.6	1196	38	3.2	16
	1.3	32432	2232	6.9	1481	136	9.2	22
	2.5	50489	2063	4.1	1579	105	6.7	32
	5.0	75075	2758	3.7	1752	100	5.7	43
	10	89922	4707	5.2	1746	9	0.5	52
	20	60208	2896	4.8	1650	35	2.1	36
pMEK1/2	0	346	29	8.4	349	13	3.9	
	0.31	11108	352	3.2	1102	41	3.7	10
	0.63	19518	817	4.2	1523	49	3.2	13
	1.3	38011	575	1.5	1812	66	3.6	21
	2.5	60152	928	1.5	2031	47	2.3	30
	5.0	102748	4020	3.9	2819	21	0.7	36
	10	143528	1480	1.0	3388	8	0.3	42
	20	163450	364	0.2	3454	17	0.5	47

Assay Components

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

Analyte	Source Species	
	MSD Capture Antibody	MSD Detection Antibody
Phospho-ERK1/2	Mouse Monoclonal	Mouse Monoclonal
Phospho-MEK1/2	Mouse Monoclonal	Rabbit Polyclonal
Phospho-STAT3	Mouse Monoclonal	Rabbit Polyclonal

Limitations of the Procedure

The following points should be noted with the MULTI-SPOT ERK-STAT3 Cascade Assay 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. Rubinfeld H, Seger R. The ERK cascade: a prototype of MAPK signaling. *Mol Biotechnol.* 2005 Oct;31(2):151-74.
2. Frémin C, Meloche S. From basic research to clinical development of MEK1/2 inhibitors for cancer therapy. *J Hematol Oncol.* 2010 Feb 11;3:8.
3. Lim CP, Cao X. Structure, function, and regulation of STAT proteins. *Mol Biosyst.* 2006 Nov;2(11):536-50.
4. Mekori YA, Gilfillan AM, Akin C, Hartmann K, Metcalfe DD. Human mast cell apoptosis is regulated through Bcl-2 and Bcl-XL. *J Clin Immunol.* 2001 May;21(3):171-4.

Appendix

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 μ L 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 μ L. 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 μ L 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 μ L).

Summary Protocol

MSD 96-well MULTI-SPOT ERK-STAT3 Cascade Assay Kit

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing the
MULTI-SPOT ERK-STAT3 Cascade Assay.

Step 1 : Block Plate and Prepare Samples

Add 150 μ L/well of blocking solution.
Incubate at room temperature with vigorous shaking (300-1000 rpm) for 1 hour.
Prepare complete lysis buffer just prior to sample dilution.
Prepare positive and negative cell lysates and keep on ice until use.

Step 2 : Wash and Add Sample

Wash the plate 3 times with 300 μ L/well of Tris Wash Buffer.
Dispense 25 μ L/well samples.
Incubate at room temperature with vigorous shaking (300-1000 rpm) for 3 hours.

Step 3 : Wash and Add Detection Antibody Solution

Wash the plate 3 times with 300 μ L/well of Tris Wash Buffer.
Dispense 25 μ L/well of 1X detection antibody solution.
Incubate at room temperature with vigorous shaking (300-1000 rpm) for 1 hour.

Step 4 : Wash and Read Plate

Wash the plate 3 times with 300 μ L/well of Tris Wash Buffer.
Dispense 150 μ L/well of 1X Read Buffer T.
Analyze plate on SECTOR Imager within 5 minutes of read buffer addition.

	1	2	3	4	5	6	7	8	9	10	11	12
A	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
B	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
C	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
D	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
E	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
F	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
G	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
H	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

	1	2	3	4	5	6	7	8	9	10	11	12
A	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
B	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
C	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
D	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
E	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
F	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
G	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
H	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>