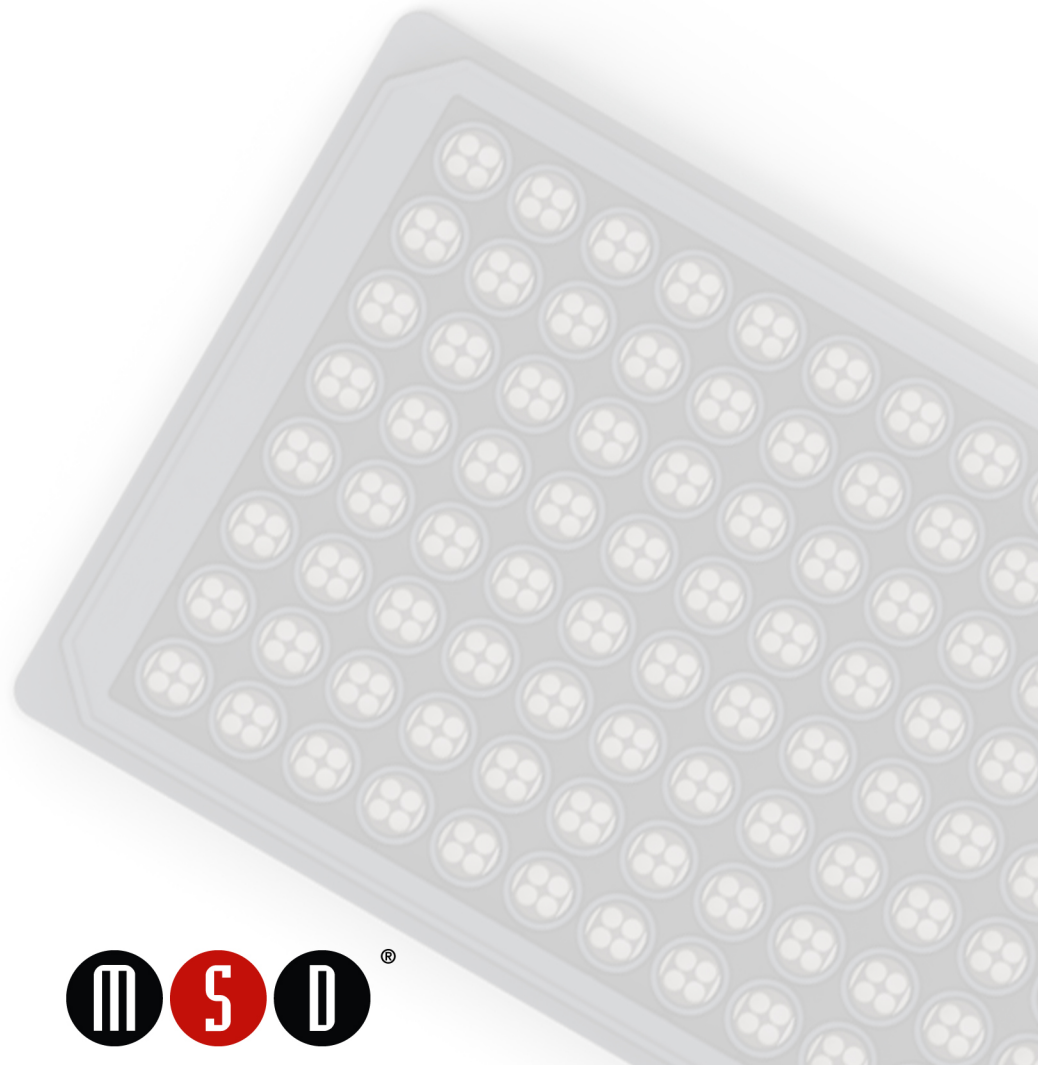


# MSD<sup>®</sup> MULTI-ARRAY Assay System

## Phospho-ERK5 (Thr218/Tyr220) Assay Base Kit

20-Plate Kit

K150IVA-3



[www.mesoscale.com](http://www.mesoscale.com)<sup>®</sup>

# MSD Phosphoprotein Assays

## Phospho-ERK5 (Thr218/Tyr220) Assay Base Kit

*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<sup>®</sup>**

A division of Meso Scale Diagnostics, LLC.

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# MSD Advantage

MESO SCALE DISCOVERY'S unique spot patterns are a hallmark of our MULTI-ARRAY<sup>®</sup> 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<sup>™</sup> labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of the MULTI-ARRAY and MULTI-SPOT<sup>®</sup> 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.

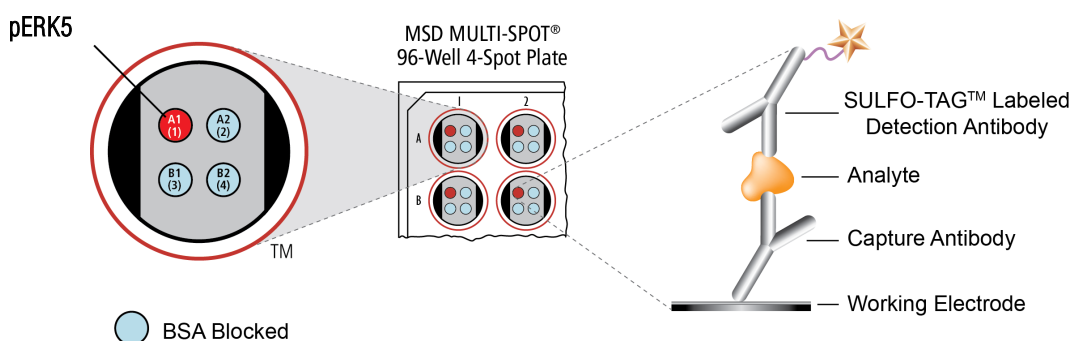
One of the advantages of MSD assays is the minimal sample volume required as compared to a traditional ELISA, which is also limited by its inability to measure more than a single analyte. With an MSD assay, up to ten different biomarkers can be analyzed simultaneously using as little as 10-25  $\mu$ 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

**ERK 5 (Extracellular signal related kinase 5)**, also known as big mitogen activated protein kinase 1 (BMPK1) and mitogen activated protein kinase 7 (MAPK7) is approximately 123 kDa and is an atypical member of the MAP kinase superfamily.<sup>1</sup> ERK5 is activated via phosphorylation on its signature Thr-Glu-Tyr motif by MEK5 and plays a role in such cellular processes as proliferation, differentiation, transcriptional regulation, and development.<sup>2</sup> ERK 5 has many similarities to ERK1/2 in terms of activation stimuli as well as downstream signaling partners, but there are also some notable differences between these two MAPKs. In vivo, ERK5 plays a role in cardiovascular development and neuronal differentiation.<sup>2</sup> Multiple MAPKs are also transcription factors and ERK5 is no exception. ERK5, unlike other MAPK family members, has a large C-terminal region which is involved in transcriptional regulation.<sup>1,3</sup> ERK 5 is normally located in both the cytoplasm and the nucleus, depending upon the cell line, but an increased concentration is seen in the nucleus upon stimulation through the MEK5 pathway.<sup>4</sup> The potential role ERK5 plays in cancer and heart disease make it an interesting target for drug development to modulate this signaling cascade.<sup>5</sup>

# 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 Phospho-ERK5 (Thr218/Tyr220) Assay is a sandwich immunoassay (Figure 1). MSD provides a plate that has been pre-coated with capture antibody for ERK5. The user adds the sample and a solution containing the detection antibody—anti-phospho-ERK5 (Thr218/Tyr220) conjugated with an electrochemiluminescent compound, MSD SULFO-TAG label—over the course of one or more incubation periods. Analyte in the sample binds to the capture antibody immobilized on the working electrode surface; recruitment of the conjugated detection antibody by bound analyte 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® 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 ERK5 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 K1501VD-3
MULTI-SPOT 96-Well 4-Spot ERK5 Plate N450IVA-1	2–8°C	20 plates
SULFO-TAG Anti-Phospho-ERK5 Antibody <sup>1</sup> (50X)	2–8°C	4 vials (375 µL ea)
Read Buffer T (4X) R92TC-3 (50 mL), R92TC-2 (200 mL)	RT	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
- 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

## Optional Material — not supplied

- Phosphoprotein Reagent Support Pack (K0000D-3)

## 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.

<sup>1</sup> Some SULFO-TAG conjugated detection antibodies may be light-sensitive, so they should be stored in the dark.

# Reagent Preparation

## Note

The instructions below will prepare the reagents needed as described in the assay protocol. All supplemental reagents (inhibitors, buffers and blocking reagents) are available for purchase in the MSD Phosphoprotein Reagent Support Pack, or alternatively can be purchased and prepared separately by the end user. Please see the enclosed assay development insert for purchasing and preparation instructions.

## Prepare Tris Wash Buffer

Dilute 10X stock of 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 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  $\mu$ L Blocker F
- 1 mL blocking solution
- 1.85 mL 1X Tris Wash Buffer

Set aside on ice.

## Prepare Complete Lysis Buffer

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

- 100  $\mu$ L Protease Inhibitor Solution (100X stock)
- 100  $\mu$ L Phosphatase Inhibitor Solution I (100X stock)
- 100  $\mu$ L Phosphatase Inhibitor Solution II (100X stock)
- 40  $\mu$ 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:

- 2.94 mL antibody dilution buffer
- 60  $\mu$ L 50X SULFO-TAG Anti-Phospho-ERK5 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 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.



# 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 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  $1 - 5 \times 10^7$  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 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  $\leq -70^\circ\text{C}$ .

## 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 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 10000 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  $\leq -70^\circ\text{C}$ .

*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-ARRAY Phospho-ERK5 (Thr218/Tyr220) 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  $\mu\text{L}$  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  $\mu\text{g}/\text{well}$  in 25  $\mu\text{L}$ . 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  $\mu$ L/well of Tris Wash Buffer. Add 25  $\mu$ L 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  $\mu$ L/well of Tris Wash Buffer. Add 25  $\mu$ L 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  $\mu$ L/well of Tris Wash Buffer. Add 150  $\mu$ L 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<sup>®</sup> 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 Plate(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 “ExportFormat” 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.*

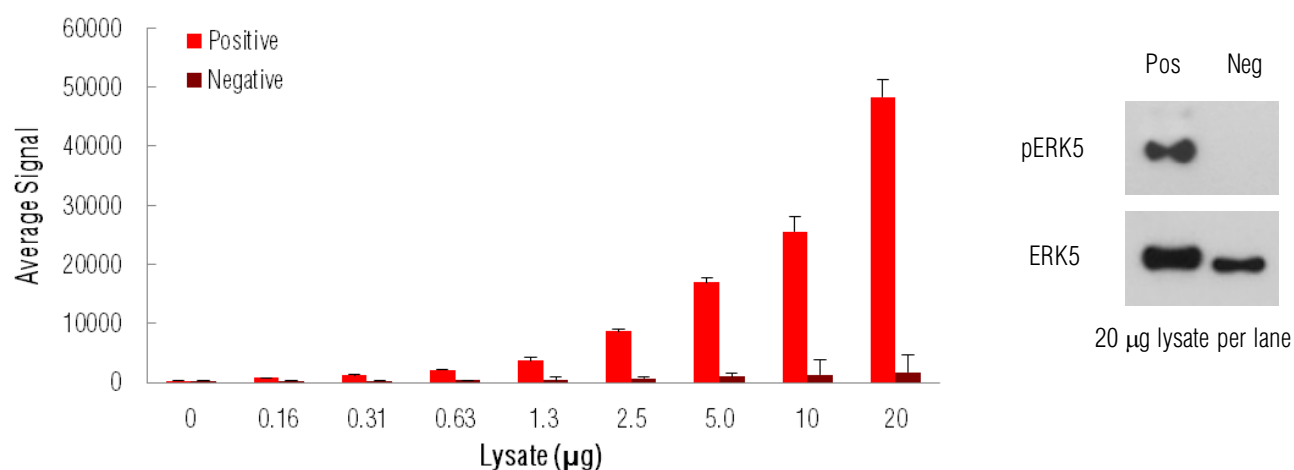
*Bubbles introduced during the read buffer addition will interfere with imaging of the plate and produce unreliable data.*

*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.*

# Typical Data

Representative results for the MULTI-ARRAY Phospho-ERK5 (Thr218/Tyr220) Assay are illustrated below. The signal and ratio values provided below are example data; individual results may vary depending upon the samples tested. Western blot analyses of each lysate type were performed with phospho-ERK5 (Thr218/Tyr220) and total ERK5 antibodies and are shown below for comparison. Confluent HeLa cell monolayers (negative) were treated with sorbitol (0.4 M, 30 minutes) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-total ERK5 antibody on one of the four spatially distinct electrodes within a well. Phosphorylated ERK5 was detected with anti-phospho-ERK5 antibody conjugated with MSD SULFO-TAG reagent.



**Figure 2:** Sample data generated with MULTI-ARRAY Phospho-ERK5 (Thr218/Tyr220) Assay. Increased signal is observed with the titration of pERK5 positive cell lysate. Signal for negative lysate remains low throughout the titration. The Phospho-ERK5 (Thr218/Tyr220) Assay provides a quantitative measure of the data obtained with the traditional Western blot.

## Lysate Titration

Data for pERK5 positive and negative HeLa cell lysates using the MULTI-ARRAY Phospho-ERK5 (Thr218/Tyr220) Assay are presented below.

Lysate (µg)	Positive			Negative			P/N
	Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
0	316	34	10.8	316	34	10.8	
0.16	795	12	1.5	323	8	2.4	2.5
0.31	1264	44	3.5	345	8	2.3	3.7
0.63	2138	44	2.1	415	33	7.8	5.2
1.3	3747	607	16.2	424	52	12.2	8.8
2.5	8670	302	3.5	735	47	6.3	12
5.0	17018	674	4.0	984	78	7.9	17
10	25606	2544	9.9	1297	21	1.6	20
20	48359	2939	6.1	1732	35	2.0	28

# Assay Components

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

Analyte	Source Species	
	MSD Capture Antibody	MSD Detection Antibody
Phospho-ERK5	Chicken Polyclonal	Rabbit Polyclonal

## Limitations of the Procedure

The following points should be noted with the MULTI-ARRAY Phospho-ERK5 (Thr218/Tyr220) 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

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2. Nishimoto S, Nishida E. MAPK signalling: ERK5 versus ERK1/2. *EMBO Rep.* 2006 Aug;7(8):782-6.
3. Akaike M, Che W, Marmarosh NL, Ohta S, Osawa M, Ding B, Berk BC, Yan C, Abe J. The hinge-helix 1 region of peroxisome proliferator-activated receptor gamma1 (PPARgamma1) mediates interaction with extracellular signal-regulated kinase 5 and PPARgamma1 transcriptional activation: involvement in flow-induced PPARgamma activation in endothelial cells. *Mol Cell Biol.* 2004 Oct;24(19):8691-704.
4. Kondoh K, Terasawa K, Morimoto H, Nishida E. Regulation of nuclear translocation of extracellular signal-regulated kinase 5 by active nuclear import and export mechanisms. *Mol Cell Biol.* 2006 Mar;26(5):1679-90.
5. Sohn SJ, Li D, Lee LK, Winoto A. Transcriptional regulation of tissue-specific genes by the ERK5 mitogen-activated protein kinase. *Mol Cell Biol.* 2005 Oct;25(19):8553-66.

# 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  $1 \times 10^4$  –  $5 \times 10^4$  cells per well and approximately  $2 \times 10^6$  cells per mL (50 – 75  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ L).

## Summary Protocol

### MSD 96-well MULTI-ARRAY Phospho-ERK5 (Thr218/Tyr220) Assay Kit

MSD provides this summary protocol for your convenience.  
Please read the entire detailed protocol prior to performing the  
MULTI-ARRAY Phospho-ERK5 (Thr218/Tyr220) Assay.

#### Step 1 : Block Plate and Prepare Samples

Add 150  $\mu$ 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  $\mu$ L/well of Tris Wash Buffer.  
Dispense 25  $\mu$ 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  $\mu$ L/well of Tris Wash Buffer.  
Dispense 25  $\mu$ L/well 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  $\mu$ L/well of Tris Wash Buffer.  
Dispense 150  $\mu$ L/well 1X Read Buffer T.  
Analyze plate on SECTOR Imager within 5 minutes of read buffer addition.





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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"/>