

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

## Akt Signaling Panel II Whole Cell Lysate Kit

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

K15177D-1  
K15177D-2  
K15177D-3



# MSD Phosphoprotein Assays

## Akt Signaling Panel II

## Whole Cell Lysate Kit

Phospho-p70S6K (Thr389), Phospho-S6RP (Ser240/244),  
Phospho-GSK-3 $\beta$  (Ser9), Phospho-Akt (Ser473)

*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>**

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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

**Akt**, also known as protein kinase B (PKB) or Rac, is a serine/threonine kinase that is of significant interest in pharmaceutical research due to its implicated role in cell growth, cell survival, cancer, and diabetes. The mammalian isoforms of Akt contain an amino-terminal pleckstrin homology (PH) domain that binds to the lipid products generated by phosphoinositide 3-kinase (PI3K). This results in the translocation of Akt to the plasma membrane, followed by a conformational change and subsequent activation of Akt by phosphorylation on Thr308 and Ser473. In an active form, Akt phosphorylates a wide variety of targets. Akt affects cell growth by the phosphorylation and inactivation of tuberlin (TSC2), an inhibitor of mTOR. In turn, activated mTOR causes increased translation through several additional downstream signaling events. Activated Akt promotes growth factor-mediated cell survival by the inhibition of apoptosis through several pathways, including the inactivation of BAD, Caspase-9, IKK $\alpha$ , and the forkhead transcription factors. An anti-apoptotic effect of Akt overexpression has been observed in breast, pancreatic, and ovarian cancer cells. Akt also regulates glycogen synthesis through the inactivation of glycogen synthase kinase 3 $\alpha$  and  $\beta$  and may play a role in glucose uptake following insulin stimulation.

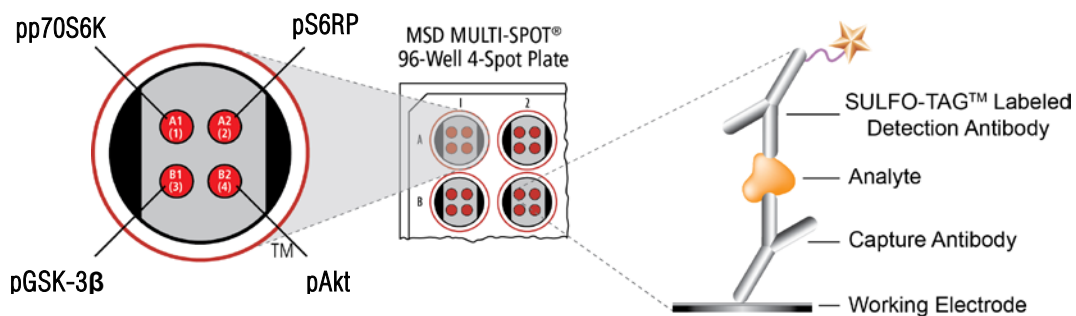
**Ribosomal protein S6 (RPS6 or S6RP)**, binds to 18s rRNA and becomes part of the small 40s ribosomal subunit. S6RP is phosphorylated on several serine residues in the carboxy terminal region, in the order 236, 235, 240, 244, 247, by p70S6 kinase (SK1). Phosphorylation of S6RP by p70S6 kinase is mediated through the mTOR/PI-3 kinase and PKC/Raf/ERK1/2 signaling cascades. These pathways are activated by growth factors and mitogens, and result in an increase in translation, specifically of 5' TOP mRNAs. The 5' TOP mRNAs contain an oligopyrimidine tract in their 5' untranslated regions and encode proteins involved in cell cycle progression, ribosomal proteins, poly(A)-binding protein and elongation factors. Ribosomal subunits containing phosphorylated S6 are more likely to be recruited in the formation of polysomes, thus resulting in an upregulation of protein synthesis and cell growth and proliferation.

**p70S6K** is a serine/threonine kinase that exists in two isoforms within the cell, a 70 kDa cytosolic protein, and an 85 kDa nuclear protein. The small ribosomal protein S6 (of the 40S subunit) is phosphorylated by active p70S6K on five serine residues. Activation of p70S6K is linked to the phosphorylation of several serine and threonine residues including Thr229, Thr389, Thr421, Ser411, and Ser424. A diverse array of proteins have been shown to play a role in p70S6K activation including PDK1, the G proteins Cdc42 and Rac1, mTOR, and the c-Raf/MEK/ERK pathway. These effectors are activated upstream by insulin, amino acids, and growth factors. In response, p70S6K exerts an effect on translation initiation, cell cycle progression, and cell survival.

**Glycogen synthase kinase-3 (GSK-3)** is a serine/threonine protein kinase that is found in two cellular isoforms -  $\alpha$  and  $\beta$ . GSK-3 has diverse cellular effects including involvement in metabolism, embryonic development and cell survival. The two isoforms are negatively regulated by phosphorylation at Ser21 (GSK-3 $\alpha$ ) and Ser9 (GSK-3 $\beta$ ) mediated by Akt in insulin signal transduction. The inhibition of GSK-3 $\alpha$  results in the dephosphorylation and activation of substrates such as glycogen synthase, eIF-2B, and C/EBP $\alpha$  causing increased protein and glycogen synthesis. Phosphorylation of GSK-3 $\beta$  at Tyr216 results in its activation and the subsequent phosphorylation of various cellular proteins including cyclin D-1 and  $\beta$ -catenin. An important member of the Wnt signaling pathway, GSK-3 plays a role in cell fate in early embryonic development. GSK-3 $\beta$  has also been implicated in the progression of Alzheimer's disease through the phosphorylation of the microtubule-associated protein tau.

# 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 Akt Signaling Panel II is a sandwich immunoassay (Figure 1). MSD provides a plate that has been pre-coated with capture antibodies for p70S6K, phospho-S6RP (Ser240/244), phospho-GSK-3 $\beta$  (Ser9), and Akt on spatially distinct spots. The user adds the sample and a solution containing the detection antibodies—anti-phospho-p70S6K (Thr389), anti-total S6RP, anti-total GSK-3 $\beta$ , and anti-phospho-Akt (Ser473) 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<sup>®</sup> 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 phospho-p70S6K (Thr389), phospho-S6RP (Ser240/244), phospho-GSK-3 $\beta$  (Ser9), and phospho-Akt (Ser473) 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		
		K15177D-1	K15177D-2	K15177D-3
MULTI-SPOT 96-Well 4-Spot Akt Signaling Panel II Plate N45177B-1	2–8°C	1 plate	5 plates	20 plates
SULFO-TAG Anti-Phospho- Akt (Ser473) Antibody <sup>1</sup> (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	4 vials (375 µL ea)
SULFO-TAG Anti-Phospho-p70S6K (Thr389) Antibody <sup>1</sup> (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	4 vials (375 µL ea)
SULFO-TAG Anti-Total GSK-3β Antibody <sup>1</sup> (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	4 vials (375 µL ea)
SULFO-TAG Anti-Total S6RP Antibody <sup>1</sup> (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 <sup>2</sup> (2%)	≤-10°C	1 vial (0.2 mL)	1 vial (0.9 mL)	4 vials (0.9 mL ea)
Blocker D-R <sup>2</sup> (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

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

<sup>2</sup> 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.

- Microcentrifuge tubes for preparing serial dilutions
- Appropriate liquid handling equipment for desired throughput, capable of dispensing 10 to 150  $\mu\text{L}$  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

- Akt Signaling Panel II Whole Cell Lysate Set (C1177-1)

## 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  $\mu$ L 2% Blocker D-M
- 30  $\mu$ 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  $\mu$ 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 PMSF in DMSO (250X stock)

The complete lysis buffer should be ice cold before use.

## Prepare Detection Antibody Solution

For one plate, combine:

- 2.76 mL antibody dilution buffer
- 60  $\mu$ L 50X SULFO-TAG Anti-Phospho-p70S6K (Thr389) Antibody (1X final concentration)
- 60  $\mu$ L 50X SULFO-TAG Anti-Total S6RP Antibody (1X final concentration)
- 60  $\mu$ L 50X SULFO-TAG Anti-Total GSK-3 $\beta$  Antibody (1X final concentration)
- 60  $\mu$ L 50X SULFO-TAG Anti-Phospho-Akt (Ser473) 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 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-SPOT Akt Signaling Panel II. The entire assay, including plate analysis on the MSD reader, can be completed in 3.5 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  $\mu\text{L}$ /well of Tris Wash Buffer. Add 25  $\mu\text{L}$  of samples per well. Seal the plate with an adhesive plate seal and incubate for 1 hour 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  $\mu\text{L}$ /well of Tris Wash Buffer. Add 25  $\mu\text{L}$  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  $\mu\text{L}$ /well of Tris Wash Buffer. Add 150  $\mu\text{L}$  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 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:

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

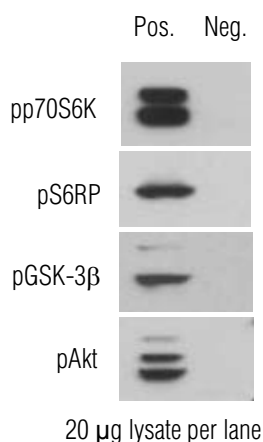
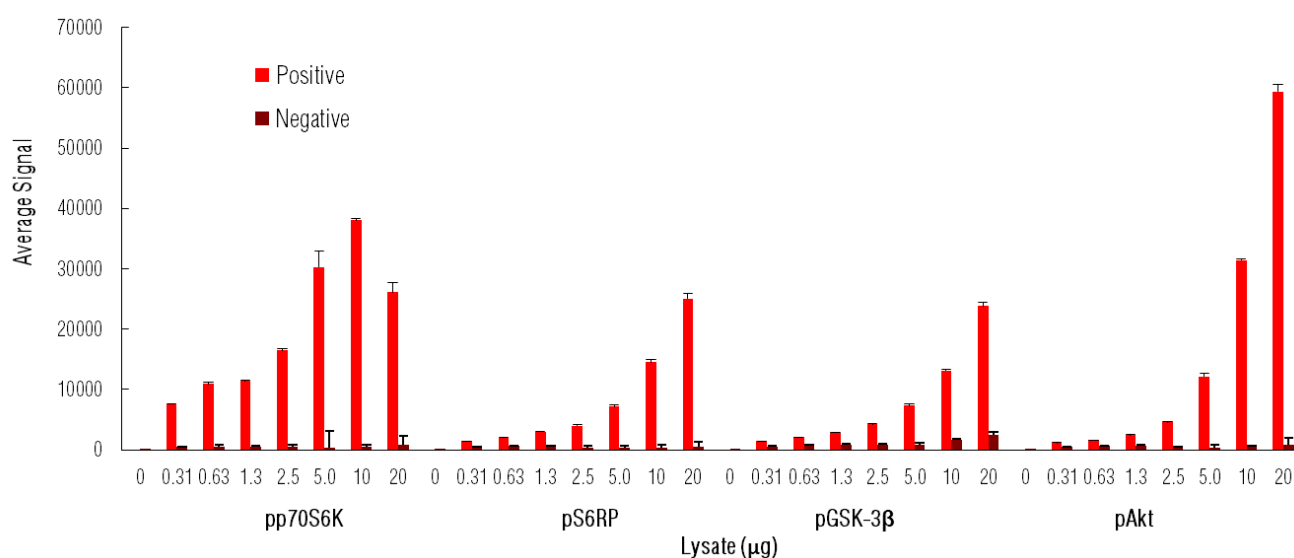
In this case, the constant in the numerator may be adjusted using the control lysates as follows:

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

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

# Typical Data

Representative results for the MULTI-SPOT Akt Signaling Panel II 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-p70S6K, phospho-S6RP, phospho-GSK-3 $\beta$ , and phospho-Akt antibodies and are shown below for comparison. Growing MCF-7 cells were treated with IGF-1 (100 nM, 20 minutes) (positive) or LY294002 (50  $\mu$ M, 2.5 hours) (negative). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-total p70S6K, anti-phospho-S6RP, anti-phospho-GSK-3 $\beta$ , and anti-total Akt antibodies on each of the four spatially distinct electrodes per well. Phosphorylated p70S6K, S6RP, GSK-3 $\beta$ , and Akt were detected with anti-phospho-p70S6K, anti-total S6RP, anti-total GSK-3 $\beta$ , and anti-phospho-Akt antibodies labeled with MSD SULFO-TAG reagent.



**Figure 2:** Sample data generated with MULTI-SPOT Akt Signaling Panel II. Increased signals for phosphorylated forms of p70S6K, S6RP, GSK-3 $\beta$ , and Akt were observed with only Akt Signaling Panel II positive cell lysate. Signals for negative lysate remained low throughout the titration. The Akt Signaling Panel II provides a quantitative measure of the data obtained with the traditional Western blot.

## Lysate Titration

Data for positive and negative MCF-7 cell lysates using the MULTI-SPOT Akt Signaling Panel II are presented below.

	Lysate (µg)	Positive			Negative			P/N
		Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
pp70S6K	0	84	4	4.2	84	4	4.2	
	0.31	7530	25	0.3	448	42	9.5	17
	0.63	10948	207	1.9	581	11	1.8	19
	1.3	11409	150	1.3	564	4	0.8	20
	2.5	16437	393	2.4	441	77	17.5	37
	5.0	30257	2763	9.1	360	36	10.0	84
	10	38034	362	1.0	490	11	2.3	78
	20	26092	1619	6.2	747	69	9.2	35
pS6RP	0	95	23	24.6	95	23	24.6	
	0.31	1388	24	1.7	497	26	5.3	2.8
	0.63	2013	13	0.6	636	35	5.5	3.2
	1.3	3027	44	1.4	570	33	5.7	5.3
	2.5	3989	213	5.3	381	28	7.4	10
	5.0	7174	264	3.7	344	21	6.0	21
	10	14510	532	3.7	360	23	6.3	40
	20	25047	885	3.5	484	24	5.0	52
pGSK-3β	0	102	18	17.6	102	18	17.6	
	0.31	1403	76	5.4	566	23	4.0	2.5
	0.63	2062	46	2.2	757	33	4.3	2.7
	1.3	2858	71	2.5	850	30	3.5	3.4
	2.5	4229	37	0.9	882	16	1.8	4.8
	5.0	7309	237	3.2	905	6	0.6	8.1
	10	13123	233	1.8	1585	113	7.1	8.3
	20	23860	510	2.1	2452	11	0.4	9.7
pAkt	0	99	10	10.0	99	10	10.0	
	0.31	1146	42	3.7	522	19	3.7	2.2
	0.63	1591	11	0.7	627	1	0.2	2.5
	1.3	2453	151	6.2	631	41	6.5	3.9
	2.5	4551	82	1.8	408	6	1.4	11
	5.0	12105	498	4.1	379	4	1.1	32
	10	31427	169	0.5	429	18	4.1	73
	20	59373	1136	1.9	848	26	3.1	70

# 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-p70S6K	Mouse Monoclonal	Mouse Monoclonal
Phospho-S6RP	Rabbit Polyclonal	Rabbit Monoclonal
Phospho-GSK-3 $\beta$	Mouse Monoclonal	Mouse Monoclonal
Phospho-Akt	Mouse Monoclonal	Mouse Monoclonal

## Limitations of the Procedure

The following points should be noted with the MULTI-SPOT Akt Signaling Panel II 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.

## Companion Products

MULTI-SPOT Akt Signaling Panel [pAkt (Ser473), pGSK-3 $\beta$ (Ser9), pp70S6K (Thr421/Ser424)]	
Kit Size	Catalog Numbers
1 plate	K15115D-1
5 plates	K15115D-2
20 plates	K15115D-3
20 plates (Base Kit)	K15115A-3
MULTI-SPOT Akt Signaling (Total Protein) Panel [Akt, GSK-3 $\beta$ , p70S6K]	
Kit Size	Catalog Numbers
1 plate	K15133D-1
5 plates	K15133D-2
20 plates	K15133D-3
20 plates (Base Kit)	K15133A-3



# References

Given below are a few references using MSD technology to measure phosphoproteins.

1. Edgar KA, Wallin JJ, Berry M, Lee LB, Prior WW, Sampath D, Friedman LS, Belvin M. Isoform-specific phosphoinositide 3-kinase inhibitors exert distinct effects in solid tumors. *Cancer Res.* 2010 Feb 1;70(3):1164-72. Epub 2010 Jan 26.
2. Grimshaw KM, Hunter LJ, Yap TA, Heaton SP, Walton MI, Woodhead SJ, Fazal L, Reule M, Davies TG, Seavers LC, Lock V, Lyons JF, Thompson NT, Workman P, Garrett MD. AT7867 is a potent and oral inhibitor of AKT and p70 S6 kinase that induces pharmacodynamic changes and inhibits human tumor xenograft growth. *Mol Cancer Ther.* 2010 May;9(5):1100-10. Epub 2010 Apr 27.
3. Gowan SM, Hardcastle A, Hallsworth AE, Valenti MR, Hunter LJ, de Haven Brandon AK, Garrett MD, Raynaud F, Workman P, Aherne W, Eccles SA. Application of meso scale technology for the measurement of phosphoproteins in human tumor xenografts. *Assay Drug Dev Technol.* 2007 Jun;5(3):391-401.

# 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-SPOT Akt Signaling Panel II Assay Kit**

MSD provides this summary protocol for your convenience.  
Please read the entire detailed protocol prior to performing the  
MULTI-SPOT Akt Signaling Panel II 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 1 hour.

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



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