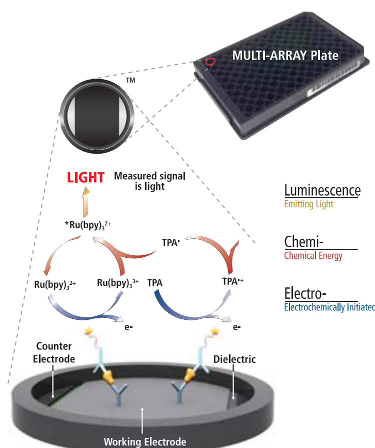
# Intracellular Phosphoproteins in Chemotherapeutic Drug Development and Diagnostic Biomarker Discovery: Akt/mTOR Signaling Pathway



Signal transduction processes are critical for the biological function of eukaryotic cells. The PI3K/Akt/mTOR pathway is one of the most potent prosurvival signaling cascades. Reversible phosphorylation of serine, threonine, and tyrosine residues is a common cell signaling mechanism in mammalian cells. Abnormal phosphorylation often leads to cancer and other severe diseases, with poor prognosis. Thus, components of the PI3K/Akt/mTOR cascade are promising targets for the design of cancer therapeutics. Several targets in the PI3K/Akt/mTOR pathway (i.e., Akt, PRAS40, GSK3b, mTOR, p70S6K, S6RP, 4EBP1, FOXO3a, FRS2, eIF4E) were examined with MSD® multiplex assays that enable quantification of phosphorylated and total protein levels to sub-microgram amounts of total protein input. MSD's rapid protocols were used to determine IC50 values in cancer cell lines as well as in peripheral blood mononuclear cells (PBMCs). MSD's recombinant Akt1 was used as a calibrator for quantification of the protein in different cell lines.

# The MSD Platform

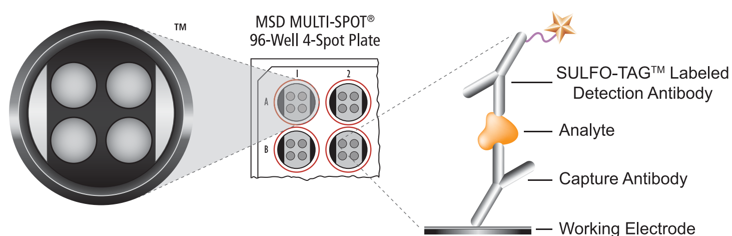
MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels, which emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.




## Electrochemiluminescence Features:

- Minimal non-specific backgrounds and strong signal responses to analyte yield high signal to background ratios
- The stimulation mechanism (electricity) is decoupled from the response (light signal)
- Proximity assay - only labels bound near the electrode surface are excited, enabling non-washed assays
- Flexibility - labels are stable, non-radioactive, and directly conjugated to biological molecules
- Emission at ~620 nm - eliminating problems with color quenching
- Signal amplification - multiple rounds of excitation and emission of each label enhance light levels and improve sensitivity
- Carbon electrode surface has 10X greater binding capacity than polystyrene well
- Surface coatings can be customized

# MSD MULTI-ARRAY Technology and MULTI-SPOT Plates




## Protocol:

1. Add 150 µL blocking solution. Incubate for 1 hour at RT.
2. Wash with TBS-T. Add 25 µL cell lysates. Incubate for 1 to 3 hours at RT.
3. Wash with TBS-T. Add 25 µL of detection antibody. Incubate for 1 hour at RT.
4. Wash with TBS-T. Add 150 µL of Read Buffer T and then read on a SECTOR® Imager.

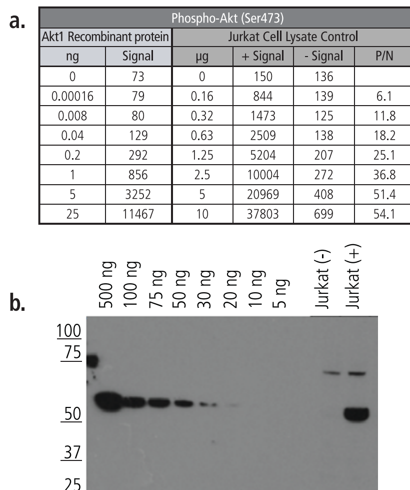
# MSD Akt1 Recombinant Protein for Clinical Applications

MSD's new Akt1 recombinant protein is phosphorylated and is designed for use with clinical samples. The calibrator and cell lysates (positive = growing, negative = LY294002 treated) were titrated in the MULTI-ARRAY Phospho-Akt (S473) and Total Akt assays and confirmed with western blots using the same detection antibodies.

**Figure 1.**

a. MSD pAkt (Ser473) assay.

b. Traditional western blot using the same pAkt(S473) antibody used in the MSD assay (cell lysate, 20 µg/lane).



a.

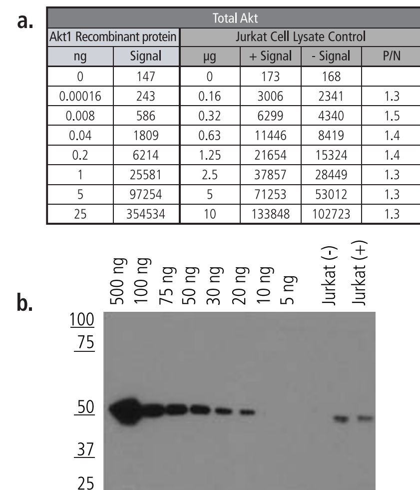
| Phospho-Akt (Ser473) | | | | | |
|---|---|---|---|---|---|
| Akt1 Recombinant protein | | Jurkat Cell Lysate Control | | | |
| ng | Signal | µg | + Signal | - Signal | P/N |
| 0 | 73 | 0 | 150 | 136 | |
| 0.00016 | 79 | 0.16 | 844 | 139 | 6.1 |
| 0.008 | 80 | 0.32 | 1473 | 125 | 11.8 |
| 0.04 | 129 | 0.63 | 2509 | 138 | 18.2 |
| 0.2 | 292 | 1.25 | 5204 | 207 | 25.1 |
| 1 | 856 | 2.5 | 10004 | 272 | 36.8 |
| 5 | 3252 | 5 | 20969 | 408 | 51.4 |
| 25 | 11467 | 10 | 37803 | 699 | 54.1 |



**Figure 2.**

a. MSD Total Akt assay.

b. Traditional western blot using the same total Akt antibody used in the MSD assay (cell lysate, 20 µg/lane).



a.

| Total Akt | | | | | |
|---|---|---|---|---|---|
| Akt1 Recombinant protein | | Jurkat Cell Lysate Control | | | |
| ng | Signal | µg | + Signal | - Signal | P/N |
| 0 | 147 | 0 | 173 | 168 | |
| 0.00016 | 243 | 0.16 | 3006 | 2341 | 1.3 |
| 0.008 | 586 | 0.32 | 6299 | 4340 | 1.5 |
| 0.04 | 1809 | 0.63 | 11446 | 8419 | 1.4 |
| 0.2 | 6214 | 1.25 | 21654 | 15324 | 1.4 |
| 1 | 25581 | 2.5 | 37857 | 28449 | 1.3 |
| 5 | 97254 | 5 | 71253 | 53012 | 1.3 |
| 25 | 354534 | 10 | 133848 | 102723 | 1.3 |

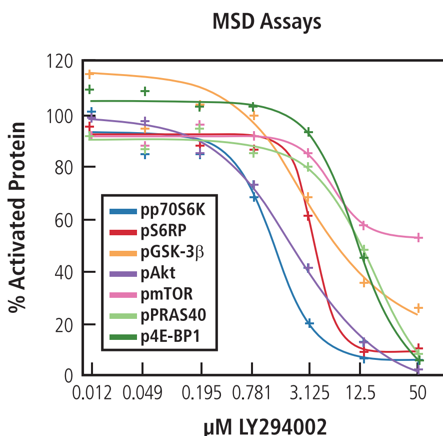


# $IC_{50}$ Measurements Obtained in a Breast Cancer Cell Line

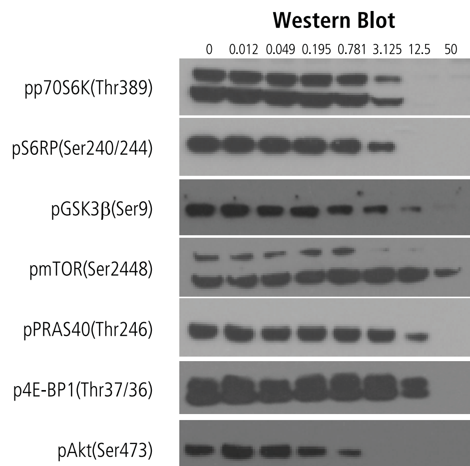
Measuring drug potency against components of the Akt signaling pathway presents a promising approach for assessing cancer therapeutics. MCF-7 cells were treated with LY294002 and IGF-1, lysed, and transferred directly from a 96-well tissue culture plate to MSD MULTI-SPOT plates. A 2-fold larger amount of lysate was used to complete traditional western blot analysis.

## LY294002 Titration (2 hr) + IGF-1 (50 nM; 20 min)

**MSD Assays**




**Western Blot**



# Human PBMC studies with phospho-Akt and phospho-PRAS40

Human PBMCs were purified from whole blood. Whole cell lysates were prepared and examined using the MSD MULTI-ARRAY Phospho-Akt (Ser473) and Phospho-PRAS40 (Thr246) Whole Cell Lysate Kits.

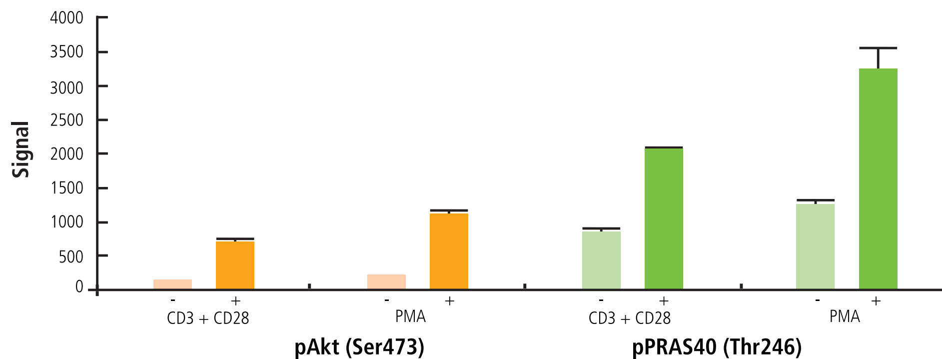



**Figure 1.** Human PBMCs were stimulated with either PMA (10 nM; 20 min.) or CD3 + CD28 antibodies (5 µg/mL each; 30 min.).

Human PBMCs were treated with serial dilutions of the PI3 Kinase inhibitor LY294002. The cells were then stimulated with either PMA (10 nM; 20 min) or CD3 + CD28 antibodies (5 µg/mL each; 30 min).

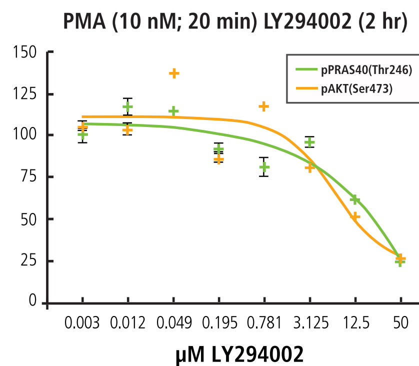



**Figure 2.** $IC_{50}$ curves for LY294002 inhibition of activated Akt and PRAS40 proteins after stimulation with PMA.

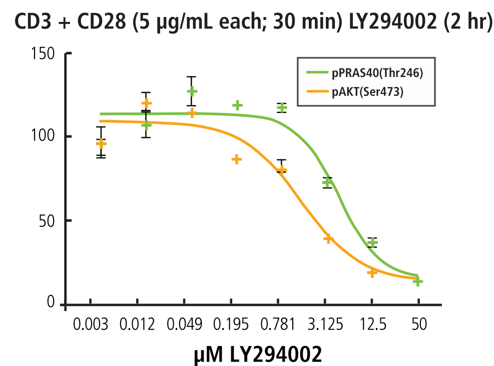
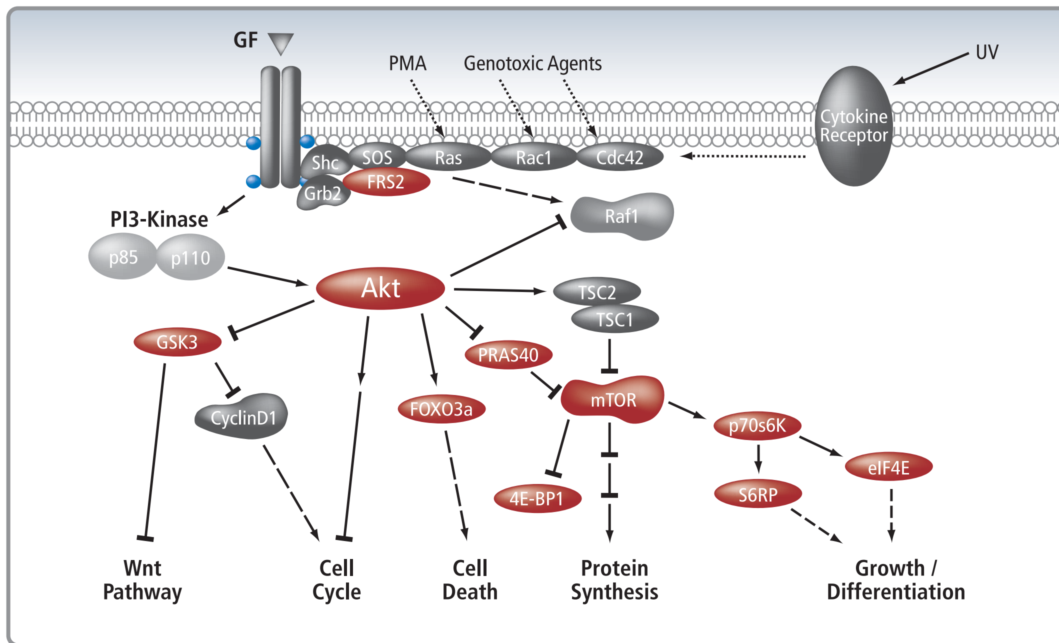



**Figure 3.** $IC_{50}$ curves for LY294002 inhibition of activated Akt and PRAS40 proteins after stimulation with a mixture of CD3 and CD28 antibodies.

# PI3K/AKT/mTOR Signaling Pathway



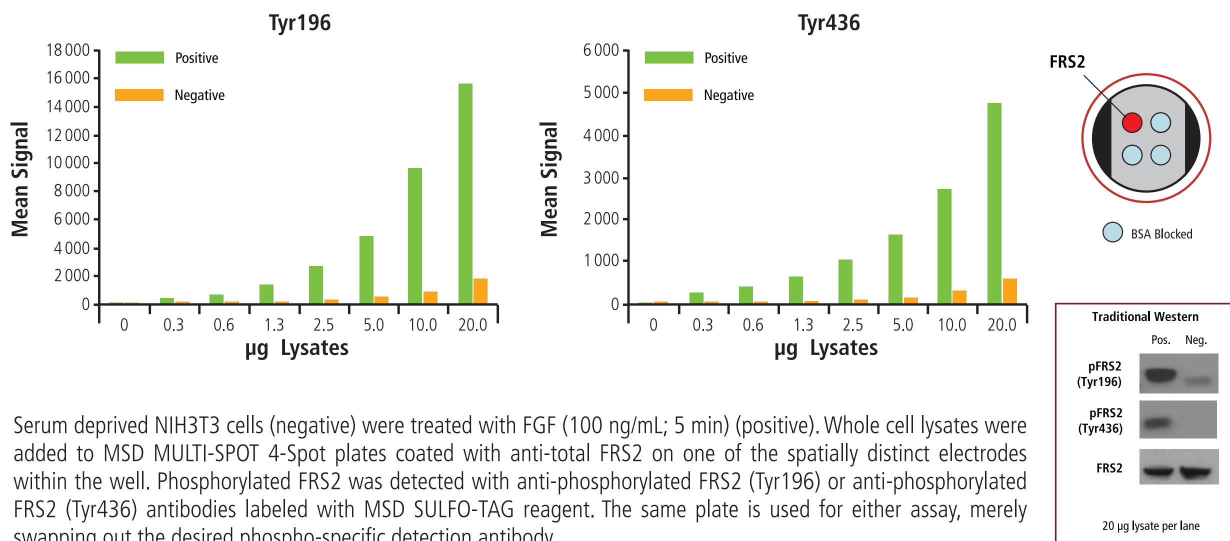

Available AKT/mTOR pathway phosphoprotein targets

# Introduction of New Target Components for the PI3K/Akt/mTOR Signaling Cascade

Transmission of growth regulatory signals from upstream cell surface receptor to its downstream target effectors in subcellular organelles provide the opportunity to examine cross-talk among different pathways (e.g., PI3K/Akt/mTOR, Ras/Raf/MEK/ERK).

- New targets shown here: FRS2 (Tyr196), FRS2 (Tyr436), total eIF4E, eIF4E (Ser209), and FOXO3a (Thr32)
- New available targets not shown here: total PRAS40, PRAS40 (Thr246), and 4E-BP1 (Thr37/46)

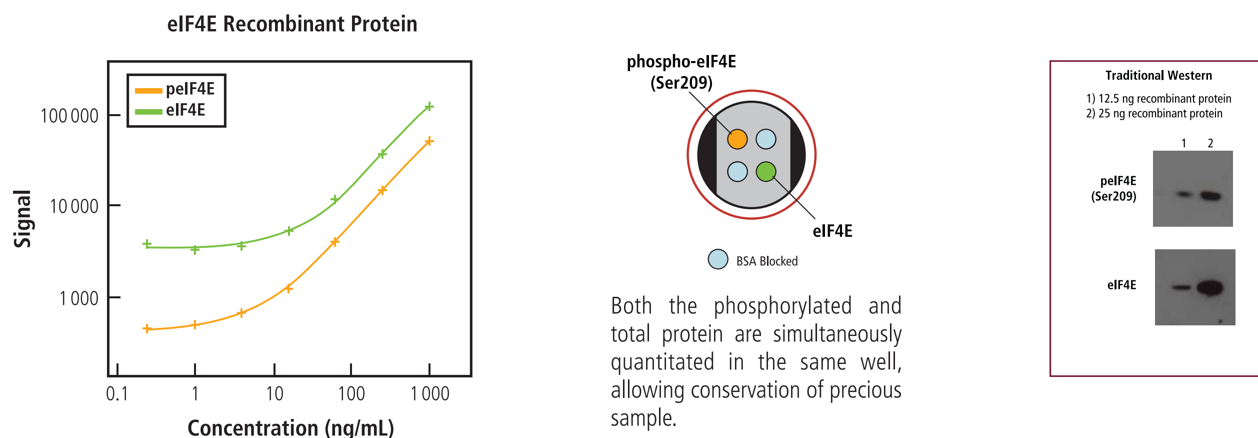
## Detection of Phospho-FRS2 (Tyr196) and (Tyr436) in a Fibroblast Cell Line




Serum deprived NIH3T3 cells (negative) were treated with FGF (100 ng/mL; 5 min) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-total FRS2 on one of the spatially distinct electrodes within the well. Phosphorylated FRS2 was detected with anti-phosphorylated FRS2 (Tyr196) or anti-phosphorylated FRS2 (Tyr436) antibodies labeled with MSD SULFO-TAG reagent. The same plate is used for either assay, merely swapping out the desired phospho-specific detection antibody.

## Detection of Phospho- (Ser209) and Total eIF4E

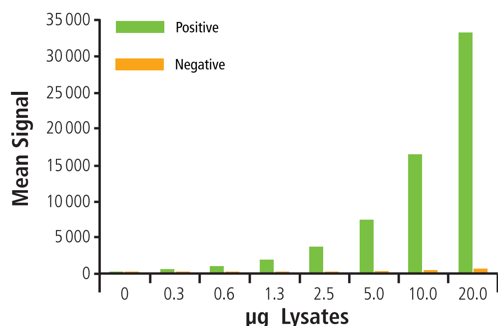
Recombinant protein (endogenously phosphorylated), was titrated on the phospho and total eIF4E MSD assay. Results agree with conventional western blot analysis using the same antibodies.



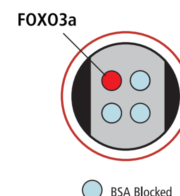

Both the phosphorylated and total protein are simultaneously quantitated in the same well, allowing conservation of precious sample.

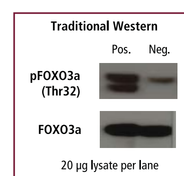
# Introduction of New Target Components for the PI3K/Akt/mTOR Signaling Cascade

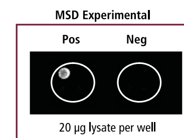
## Forkhead Transcription Factor (Phospho-FOXO3a (Thr32)) Monitored in Breast Cancer Cells.




| Lysate (μg) | Positive | | | Negative | | | P/N |
|---|---|---|---|---|---|---|---|
| | Average | StdDev | %CV | Average | StdDev | %CV | |
| 0 | 159 | 7 | 4.4 | 163 | 1 | 0.4 | |
| 0.3 | 587 | 18 | 3.0 | 168 | 3 | 1.7 | 3.5 |
| 0.6 | 980 | 57 | 5.8 | 171 | 8 | 4.6 | 5.7 |
| 1.3 | 1850 | 66 | 3.6 | 191 | 9 | 4.8 | 9.7 |
| 2.5 | 3677 | 197 | 5.3 | 228 | 7 | 3.1 | 16.1 |
| 5.0 | 7431 | 255 | 3.4 | 271 | 17 | 6.3 | 27.4 |
| 10.0 | 16458 | 853 | 5.2 | 372 | 6 | 1.5 | 44.2 |
| 20.0 | 33328 | 1013 | 3.0 | 613 | 33 | 5.4 | 54.4 |




Growing MCF-7 cells were treated with IGF-1 (100 nM, 20 min) (positive) or LY294002 (50 μM, 2.5 hr) (negative). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-total FOXO3a on one of the spatially distinct electrodes within the well. Phosphorylated FOXO3a was detected with anti-phosphorylated FOXO3a (Thr32) antibody labeled with MSD SULFO-TAG reagent.

# Conclusions

- MSD has developed a suite of multiplex assays that enables mapping of the PI3K/Akt/mTOR pathway using rapid protocols that yield highly quantitative results. Detection limits in several cancer cell lines are in the the sub-microgram ranges affording compatibility with 96-well, and in some cases, 384-well cultures.
- Antagonistic drug potency can be measured in breast cancer cells as well as in whole blood PBMCs.
- Several new assays are available for examining targets of the Akt signaling cascade: pFRS2 (Tyr196 and Tyr436), pFOXO3a (Thr32), peIF4E (Ser209), eIF4E, p4E-BP1 (Thr37/46), pPRAS40 (Thr246), and PRAS40.



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