Analyzing cross-talk between Ras-Raf-MEK-ERK and PI3K-Akt Signaling Pathways: A highly sensitive multiplex assay for simultaneous determination of Phospho-Erk and Phospho-Akt

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Abstract

The Ras-Raf-MEK-ERK and phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathways are synergistically regulated and have been shown to cross-talk in human breast cancer cell lines[1]. High PI3K/Akt activity has critical roles in prevention of apoptosis and regulation of cell cycle progression, whereas high Raf activity induces growth arrest and differentiation in these cells. Here, we present a sensitive method for analyzing cross-talk between these pathways using their selective activation. LY294002 (Ly) treatment of Jurkat cells completely abolished phosphorylated Akt while leaving its total levels unaltered. Treatment of these same cells with Phorbol 12-myristate 13-acetate (PMA), a differentiation-inducing stimulus, potently induced phosphorylation of T202/Y204;T185/Y187-Erk1/2 and, as expected, only weakly phosphorylated S473-Akt. Interestingly, using a combination of PMA activation and LY-treatment, Jurkat cells had high levels of phosphorylated Erk, and significant amounts of phosphorylated Akt. Since Ly demonstrably shut down the AKT pathways, this phosphorylation of Akt was most likely the result of cross talk, probably with the Ras pathway and was quantifiable in a high throughput format. These measurements are highly relevant to an understanding of cellular transformations and may be of use in areas like tissue engineering. The high throughput format of these targets will facilitate drug discovery efforts.

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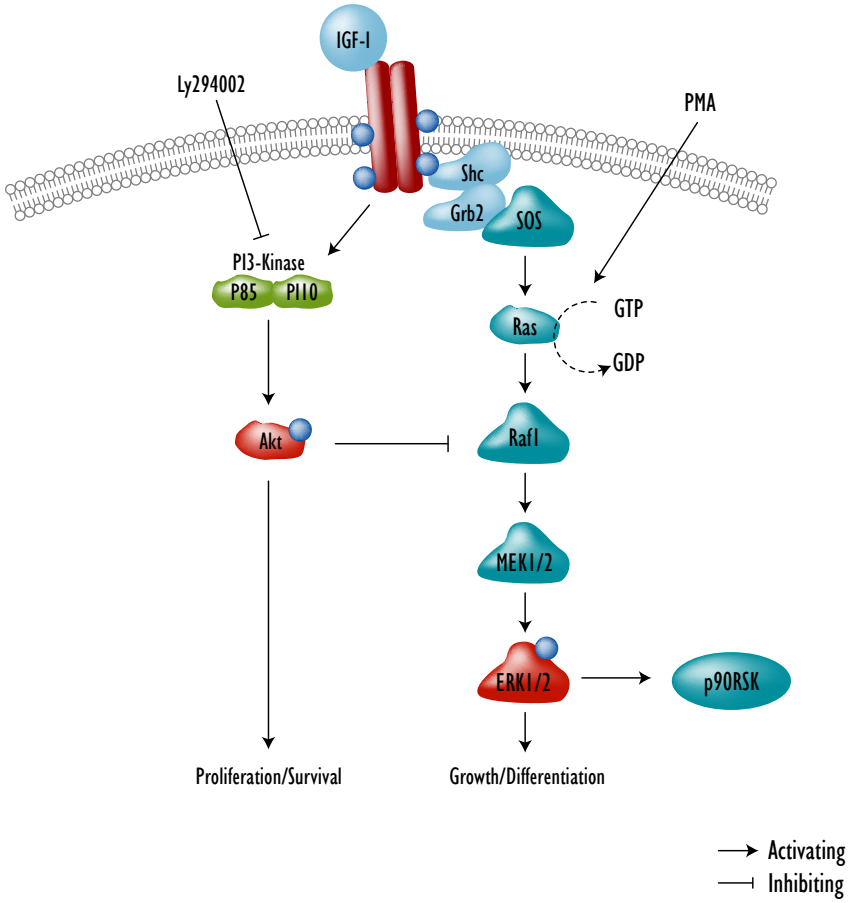
❷ Interactions between PI3K/Akt and Raf/MEK/Erk pathways



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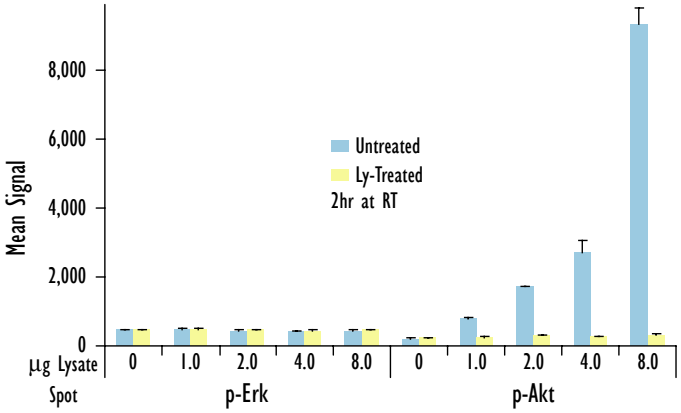
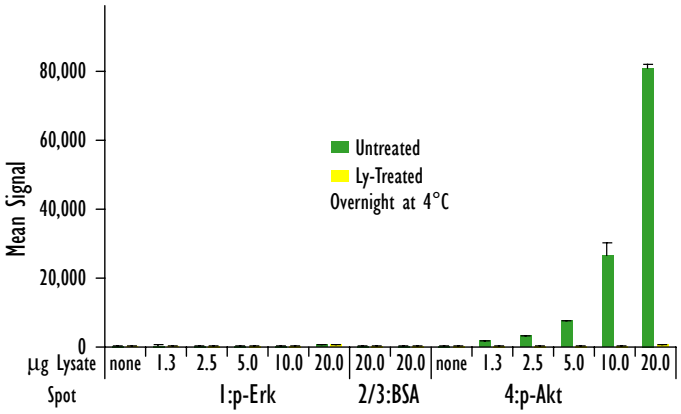
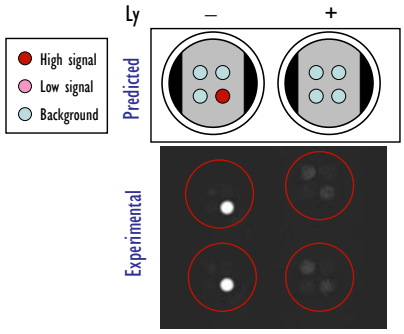
❸ Phosphoprotein Duplex Panel: Specific detection of Phospho-Akt in Jurkat whole Cell Lysates

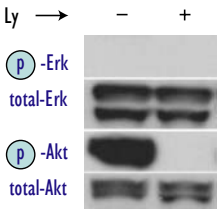




Overnight at 4°C

Spot	Lysate µg	Untreated Ave	Untreated %CV	Ly-Treated Ave	Ly-Treated %CV	S-B	S/B
1:pErk	20.0	617	5	602	4	15	1
2:BSA	20.0	280	3	265	6	15	1
3:BSA	20.0	283	1	243	1	40	1
4:pAkt	none	290	4	265	3	25	1
4:pAkt	1.3	1,771	12	404	2	1,367	4
4:pAkt	2.5	3,291	0	424	5	2,867	8
4:pAkt	5.0	7,667	1	407	1	7,260	19
4:pAkt	10.0	26,523	14	505	5	26,018	53
4:pAkt	20.0	80,705	1	681	23	80,024	119





Logarithmically growing Jurkat cells were treated with (+/-) Ly Inhibitor. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well High Bind plates coated with capture antibody at two of the four spatially distinct electrodes per well. Phosphorylated proteins were detected with MSD SULFO-TAG detection antibodies.

As expected, high phosphorylation of Akt and not of Erk was observed in the untreated Jurkat cells. The phosphorylation of Akt was completely abolished with the Ly294002 treatment.

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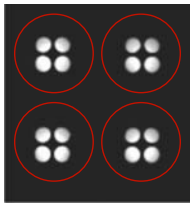
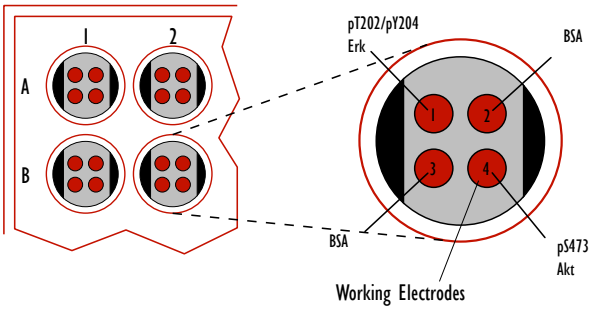
❹ MSD MULTI-ARRAY™ Technology and MULTI-SPOT™ Plates

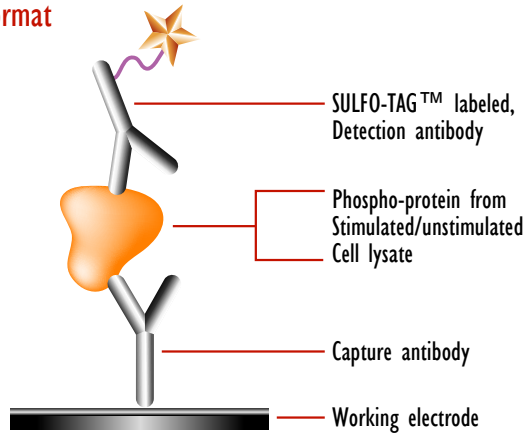
96-Well Plate



4 Spot 96-Well Plates



Assay Format



1. MULTI-SPOT 4 Spot 96-Well Plates precoated with capture antibodies are blocked with 200µL of MSD Blocker-A for 1.5h and washed with TBS.
2. 25µL of cell lysates are added to the wells and incubated for 2h at RT or overnight at 4°C with shaking, followed by washing with TBS.
3. 25µL MSD SULFO-TAG labeled antibodies (in 1% MSD-Blocker-A in TBS) are added to the wells and incubated for 1.5 h with shaking, followed by washing with TBS.
4. 150µL MSD Read Buffer T (with surfactant) are added to the wells and analyzed on the SECTOR 6000 instrument.

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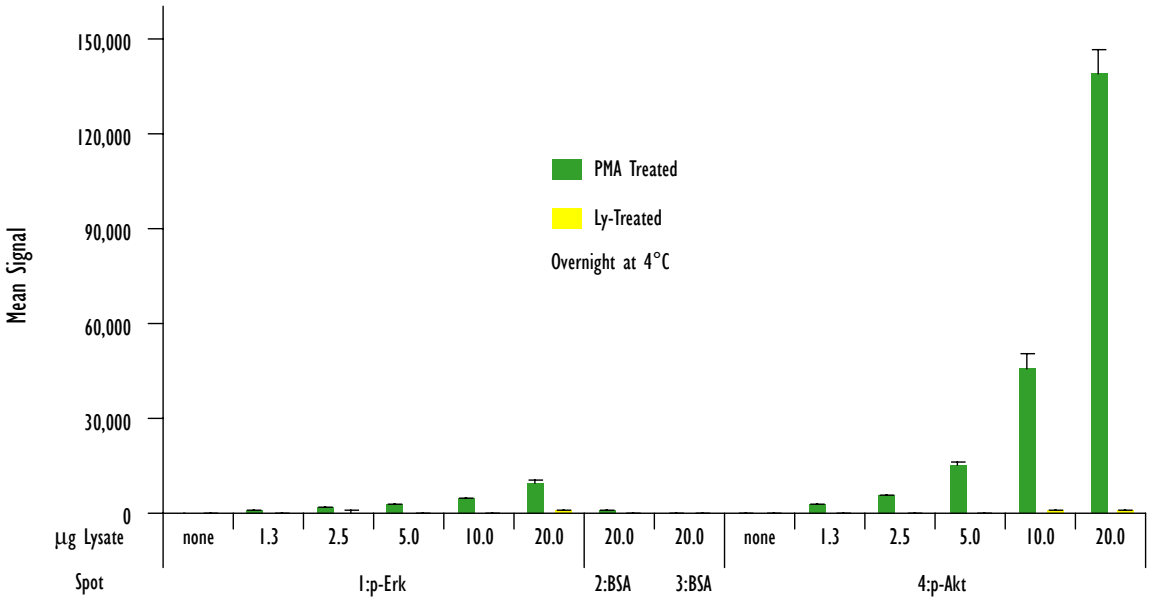
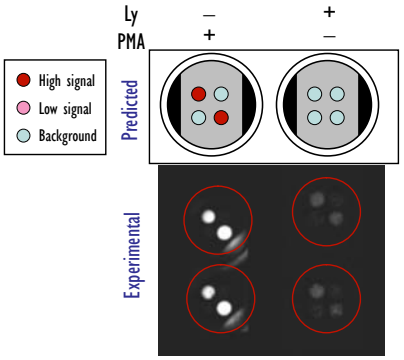
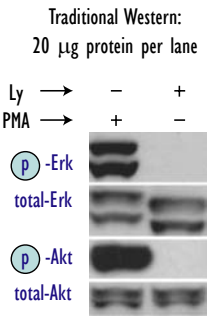
❺ Phosphoprotein Duplex Panel: Phospho-Erk and Phospho-Akt in Jurkat whole Cell Lysates



Overnight at 4°C

Spot	Lysate µg	PMA-Treated Ave	PMA-Treated %CV	Untreated[B1] Ave	Untreated[B1] %CV	S/B1	Ly-Treated[B2] Ave	Ly-Treated[B2] %CV	S/B2
1:pErk	none	434	5	402	5	1.1	401	6	1
1:pErk	1.3	1,277	2	508	14	2.5	465	0	3
1:pErk	2.5	1,716	3	459	16	3.7	473	3	4
1:pErk	5.0	2,715	6	418	3	6.5	454	1	6
1:pErk	10.0	4,825	6	467	5	10.3	470	0	10
1:pErk	20.0	9,260	10	617	5	15.0	602	4	15
2:BSA	20	495	66	280	3	1.8	265	6	2
3:BSA	20	307	7	283	1	1.1	243	1	1
4:pAkt	none	297	7	290	4	1.0	265	3	1
4:pAkt	1.3	2,715	11	1,771	12	1.5	404	2	7
4:pAkt	2.5	5,815	2	3,291	0	1.8	424	5	14
4:pAkt	5.0	15,224	8	7,667	1	2.0	407	1	37
4:pAkt	10.0	45,989	9	26,523	14	1.7	505	5	91
4:pAkt	20.0	139,479	5	80,705	1	1.7	681	23	205





Logarithmically growing Jurkat cells were treated with Ly-inhibitor or PMA. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well High Bind plates coated with capture antibody at two of the four spatially distinct electrodes per well. Phosphorylated proteins were detected with MSD SULFO-TAG detection antibodies.

Erk1/2 was strongly activated by PMA treatment (about 15 fold). Compared to this, Akt was activated to a lower extent (about 2 fold), similar to that shown earlier in MCF-7 cells[1].

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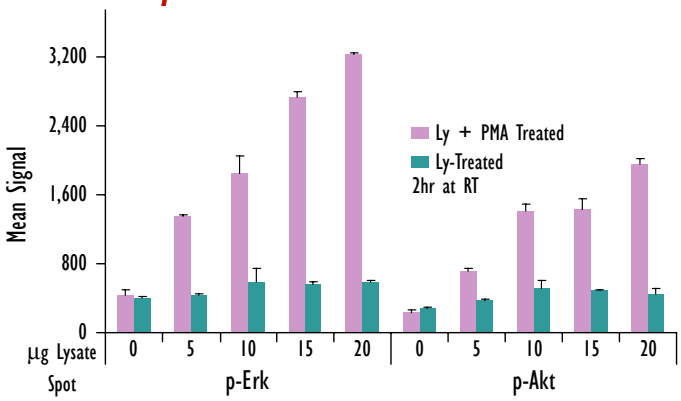
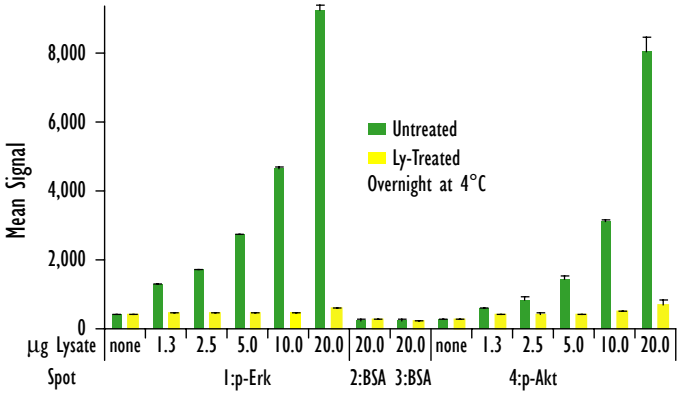
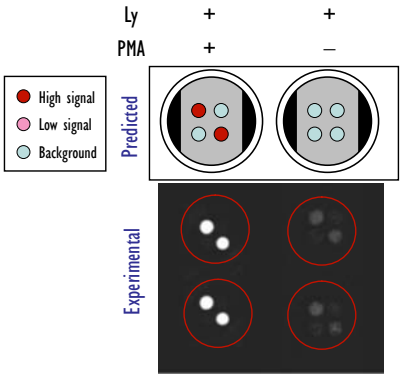
6 Phosphoprotein Duplex Panel: Phospho-Erk and Phospho-Akt in Jurkat whole Cell Lysates

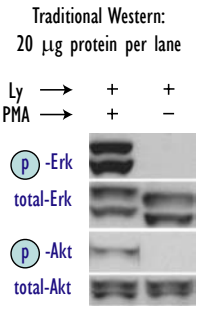




Overnight at 4°C

Spot	Lysate µg	Ly+PMA-Treated Ave	Ly+PMA-Treated %CV	Ly-Treated Ave	Ly-Treated %CV	S/B	S/B
1:pErk	none	438	1	401	6	37	1
1:pErk	1.3	1,312	0	465	0	847	3
1:pErk	2.5	1,729	0	473	3	1,256	4
1:pErk	5.0	2,744	0	454	1	2,291	6
1:pErk	10.0	4,673	1	470	0	4,204	10
1:pErk	20.0	9,291	2	602	4	8,690	15
2:BSA	20.0	247	7	265	6	-18	1
3:BSA	20.0	251	5	243	1	9	1
4:pAkt	none	292	3	265	3	28	1
4:pAkt	1.3	613	3	404	2	210	2
4:pAkt	2.5	822	11	424	5	398	2
4:pAkt	5.0	1,431	7	407	1	1,024	4
4:pAkt	10.0	3,106	1	505	5	2,601	6
4:pAkt	20.0	8,046	6	681	23	7,366	12





Logarithmically growing Jurkat cells were treated with Ly Inhibitor followed by (+/-) PMA. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well High Bind plates coated with capture antibody at two of the four spatially distinct electrodes per well. Phosphorylated proteins were detected with MSD SULFO-TAG detection antibodies.

Although Ly-treatment completely abolished Akt, the addition of PMA to these Ly pre-treated cells induced phosphorylation of Akt to a small but significant level (compared to high amounts in untreated cells). The phosphorylation of Erk1/2 induced by PMA was not affected by the Ly pre-treatment.

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7 Conclusion

We present highly specific, multiplexed assays for dissecting the activation of phosphorylated proteins in the Erk and Akt pathways in a single well. In Jurkat cells, PMA treatment significantly elevated (to about 15 fold) phosphorylation of T202/Y204 Erk1/2 compared to that for untreated cells. LY294002, a PI3K inhibitor, treatment rendered undetectable endogenous levels of phospho-Akt (S473) but, as expected, did not reduce the level of PMA induced Erk1/2 activation, confirming that PI3-K and MAPK pathways are independent. PMA also induced Akt phosphorylation in the absence of PI3K inhibition in Jurkat cells, similar to that shown in MCF-7 cells[1]. Phorbol esters are also known not to induce Akt-dependent Raf inhibition, thus strong Erk phosphorylation observed here is not affected by the Akt activation. Interestingly, in the LY294002 treated Jurkat cells, PMA induced small but significant phosphorylation of Akt-S473, that was detectable in our duplex assay. Where several past reports show interactions between Raf-MEK-Erk and PI3-Akt signaling pathways, our results show quantifiable modulation of Akt activity during the PMA activation of Erk1/2, possibly the result of cross-talk between the two pathways or a mechanism of PMA action that needs further evaluation. The methods we present are general, demonstrating that multiple phosphoprotein members of signaling pathways can be assayed simultaneously in a single well in short (2 hr) or long (overnight) incubation periods, using specific antibodies immobilized on MSD MULTI-SPOT plates. The MULTI-ARRAY technology-based assays are useful supplements to gold-standard methods like western blots because these versatile assays are highly quantitative and easily automated, are suitable for HTS, and save time and labor compared to existing techniques.

References:

1. Regulation of Raf-Akt Cross-talk, Moelling K, Schad K, Bosse M, Zimmermann S, Schweneker M, J Biol Chem. 277, p31099, 2002.