Multiplex Arrays to Measure phospho-p38, phospho-Akt and phospho-p53

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Abstract

Reversible phosphorylation of target proteins mediated by protein kinases is involved in nearly all regulatory events in cells. Misregulations or mutations within the members of this family of enzymes, or of their upstream partners, are cause or consequence of many disease states. Phosphoproteins are traditionally studied via western blots, immunoprecipitation, mass spectrometry or ^{32}P incorporation, but these methods are time consuming and not adaptable to high throughput requirements. We have developed a highly sensitive and specific multiplex assay to detect phosphoproteins directly in 96-well MULTI-SPOT™ plates using Meso Scale Discovery's patterned array technology. Data are shown for the simultaneous detection of three key phosphoproteins, phospho-p38 (MAPK) in UV-irradiated NIH3T3 and HT29 cells, phospho-p53 in UV-irradiated HT29 cells, and phospho-Akt in untreated but not in the Ly inhibitor-treated Jurkat cells. Sensitivity and specificity of these assays is shown and compared to western blotting.

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❷ MSD MULTI-ARRAY™ and MULTI-SPOT™ Plates

Instrument Features

- Highly sensitive imaging detection systems
- Single and multiplex plate formats
- SECTOR Imager 6000 designed for high-throughput screening (HTS)
- Rapid read times
- SECTOR Imager 6000 or SECTOR PR 100 instruments ideal for assay development
- Electrochemiluminescence (ECL) detection

SECTOR™ PR 100 Reader

SECTOR™ Imager 6000

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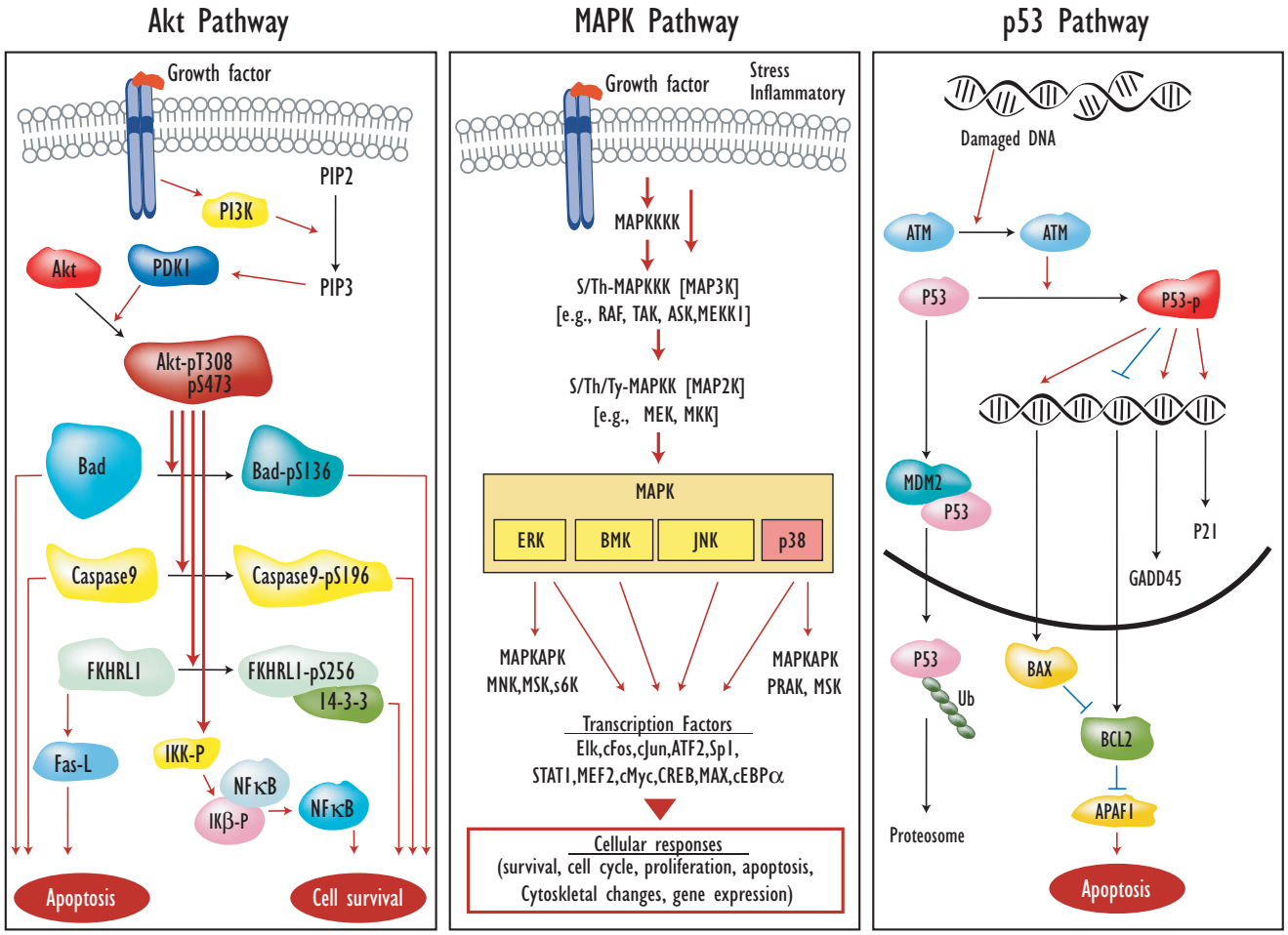
❸ Phosphoprotein Multiplex Panel: Phospho-p38, Phosphspho-p53 in NIH3T3 whole Cell Lysates



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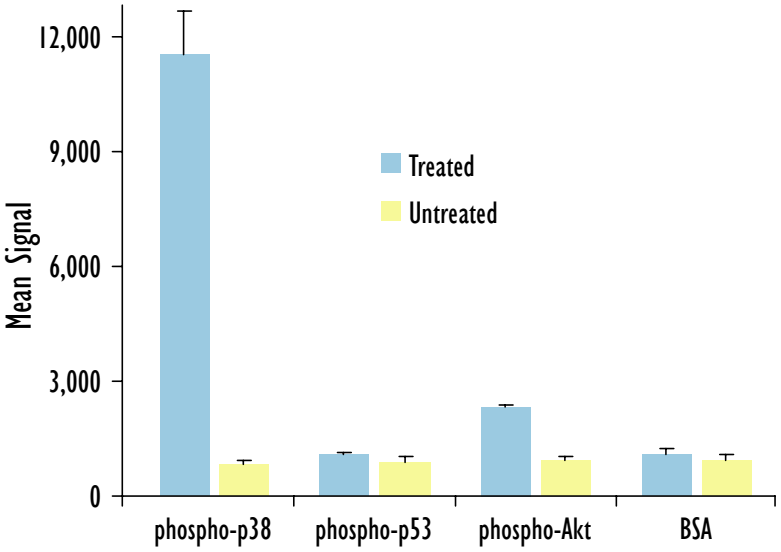
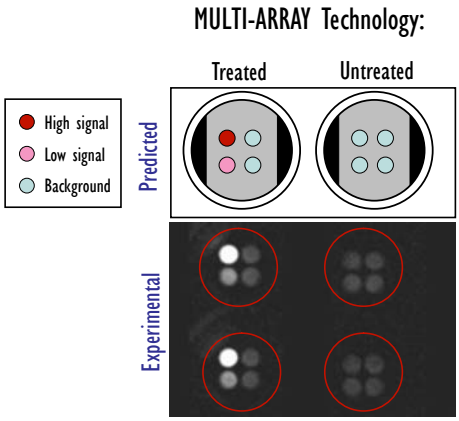
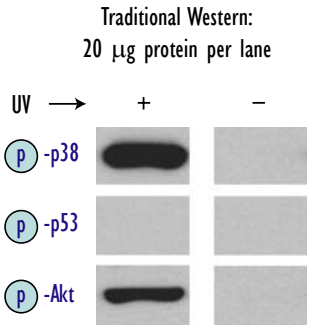
4 Phosphoprotein Multiplex Panel: Phospho-p38, Phospho-Akt and Phospho-p53 in NIH3T3 whole Cell Lysates







	Treated			Untreated			S-B	S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		
phospho-p38	11,499	1,158	10	818	130	16	10,681	14.1
phospho-p53	1,075	62	6	902	156	17	174	1.2
phospho-Akt	2,338	25	1	906	107	12	1,432	2.6
BSA	1,099	115	10	913	165	18	187	1.2

Media was removed from logarithmically growing NIH3T3 cells, followed by UV irradiation at 40mJ/cm^2. Whole cell lysates (~ 20 µg protein) were added to MSD MULTI-SPOT 4 Spot 96-well High Bind plates coated with capture antibody on three of the four spatially distinct electrodes per well (BSA was coated onto the fourth electrode in each well). Phosphorylated proteins were detected with MSD SULFO-TAG detection antibodies.

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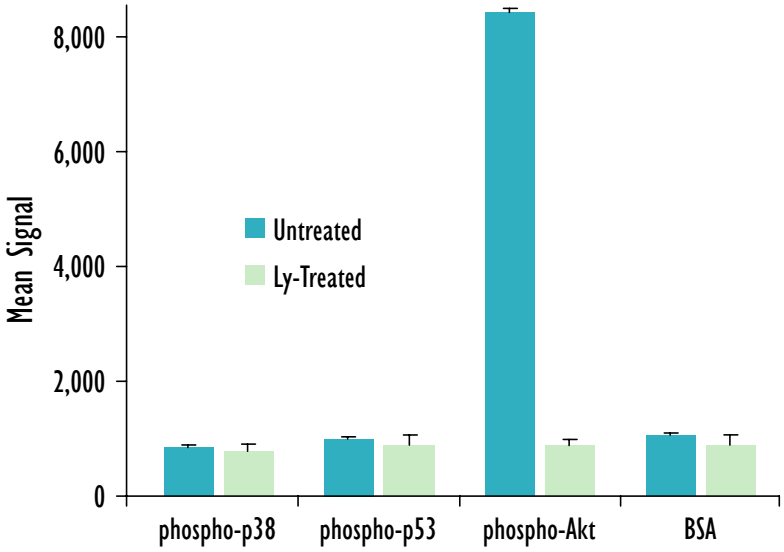
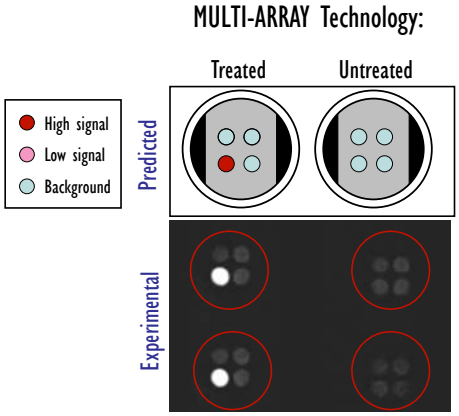
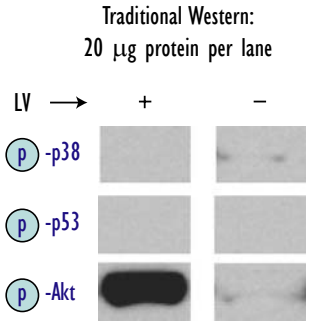
5 Phosphoprotein Multiplex Panel: Phospho-p38, Phospho-Akt and Phospho-p53 in Jurkat whole Cell Lysates







	Unreated			Ly-Treated			S-B	S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		
phospho-p38	834	38	5	797	131	16	37	1.0
phospho-p53	1,001	23	2	903	162	18	99	1.1
phospho-Akt	8,406	89	1	885	117	13	7,521	9.5
BSA	1,071	12	1	872	200	23	199	1.2

Logarithmically growing Jurkat cells were treated with Ly Inhibitor. Whole cell lysates (~ 20 µg protein) were added to MSD MULTI-SPOT 4 Spot 96-well High Bind plates coated with capture antibody on three of the four spatially distinct electrodes per well (BSA was coated onto the fourth electrode in each well). Phosphorylated proteins were detected with MSD SULFO-TAG detection antibodies.

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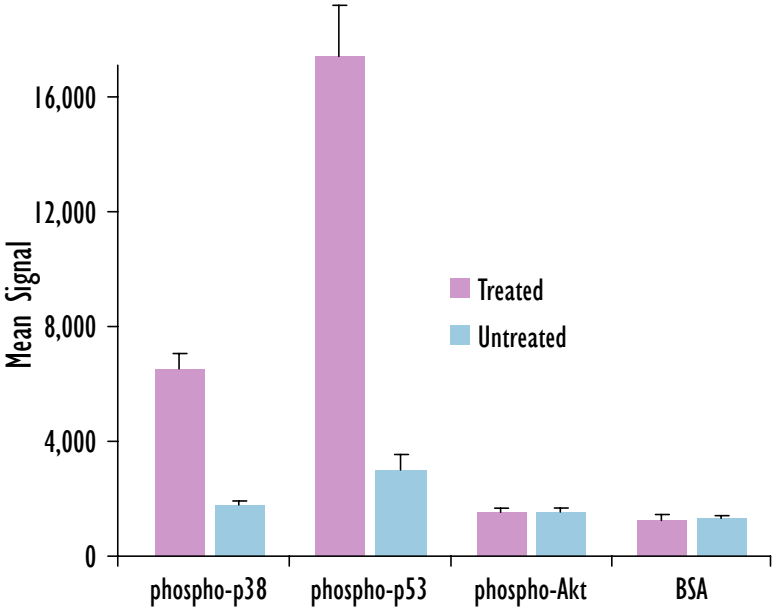
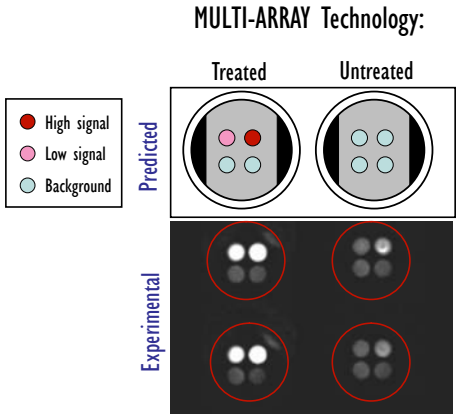
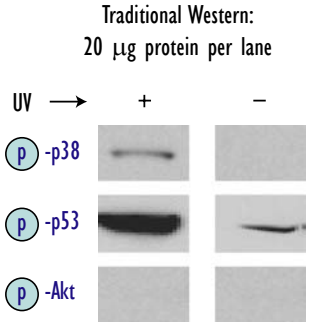
6 Phosphoprotein Multiplex Panel: Phospho-p38, Phospho-Akt and Phospho-p53 in HT-29 whole Cell Lysates







	Treated			Untreated			S-B	S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		
phospho-p38	6,553	510	8	1,769	147	8	4,784	3.7
phospho-p53	17,372	1,793	10	3,002	563	19	14,370	5.8
phospho-Akt	1,503	178	12	1,544	101	7	-41	1.0
BSA	1,215	220	18	1,291	102	8	-75	0.9

Media was removed from logarithmically growing HT29 cells, followed by UV irradiation at 40mJ/cm^2. Whole cell lysates (~ 10 µg protein) were added to MSD MULTI-SPOT 4 Spot 96-well High Bind plates coated with capture antibody on three of the four spatially distinct electrodes per well (BSA was coated onto the fourth electrode in each well). Phosphorylated proteins were detected with MSD SULFO-TAG detection antibodies.

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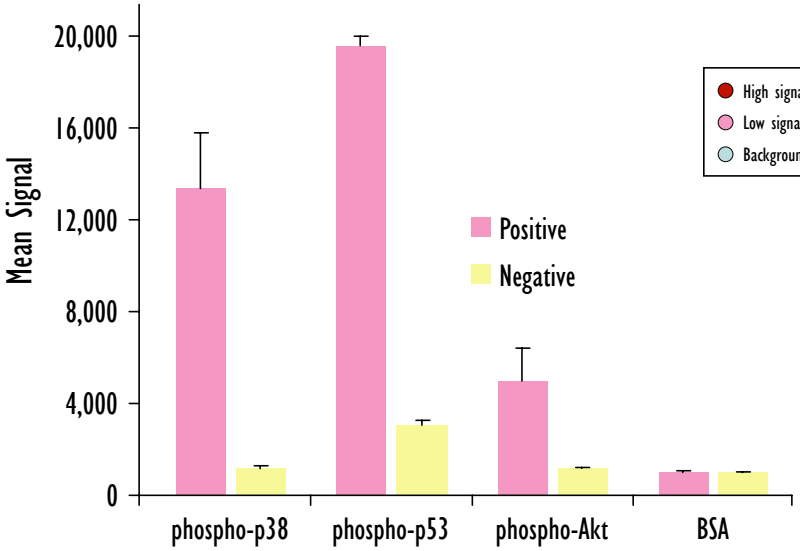
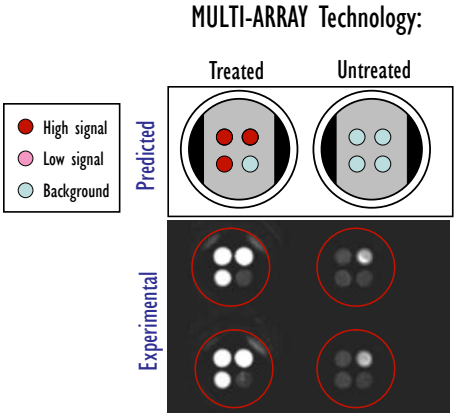
7 Phosphoprotein Multiplex Panel: Phospho-p38, Phospho-Akt and Phospho-p53 in Combined NIH3T3 + HT-29 + Jurkat whole Cell Lysates





	Positive			Negative			S-B	S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		
phospho-p38	13,359	2,383	18	1,158	68	6	12,201	11.5
phospho-p53	19,539	480	2	3,099	171	6	16,441	6.3
phospho-Akt	4,954	1,432	29	1,131	49	4	3,823	4.4
BSA	1,015	43	4	963	57	6	52	1.1

Logarithmically growing cells were treated (NIH3T3 and HT29 with UV, Jurkat with Ly-inhibitor). Combined Whole cell lysates (positives= UV-treated NIH3T3, HT29 + untreated Jurkat; Negatives = untreated NIH3T3, HT29 and Ly-inhibitor treated Jurkat) were added to MSD MULTI-SPOT 4 Spot 96-well High Bind plates coated with capture antibody on three of the four spatially distinct electrodes per well (BSA was coated onto the fourth electrode in each well). Phosphorylated proteins were detected with MSD SULFO-TAG labeled detection antibodies.

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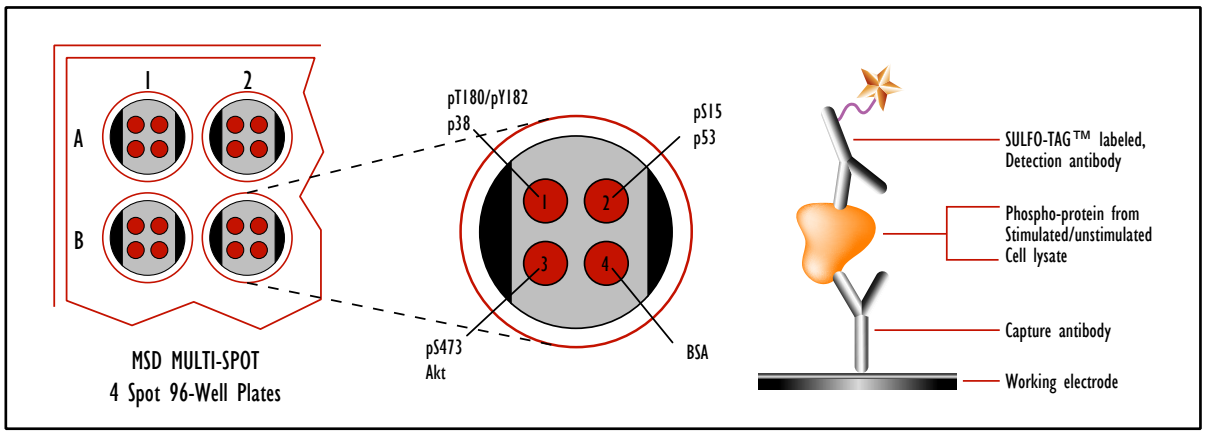
8 Assay Format



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 wash buffer (25 mM Tris pH 7.4, 0.004% Triton).
2. 25µL of cell lysates are added to the wells and incubated for 2h with shaking, followed by washing with wash buffer.
3. 25µL MSD SULFO-TAG labeled antibodies are added to the wells and incubated for 1.5h with shaking, followed by washing with Wash buffer.
4. 150µL MSD Read Buffer T (with surfactant) are added to the wells and analyzed on the SECTOR 6000 instrument.

9 Conclusion

1. We have developed highly specific multiplexed assays for simultaneously determining the phosphorylated proteins of MAPK, Akt and p53 pathways in a single well.
2. Multiple phosphoprotein members of signaling pathways can be assayed simultaneously in a single well by using specific antibodies immobilized on a MSD MULTI-SPOT plates. The MULTI-ARRAY Technology based assay can be readily adapted to any protein for which antibodies are available.
3. The assays are specific and afford high throughput replacements to the gold-standard methods like western blots. Compared to western blots, smaller numbers of cell equivalents are sufficient, further increasing the overall efficiency of the assays.
4. The assays can be easily automated, and are suitable for HTS.
5. The assays are versatile and save time and labor compared to existing techniques.

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