MULTI-SPOT™ Assays: Detection of Multiple Phosphoproteins in Whole Cell Lysates in a Single Well

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❶ Abstract

We have developed multiplexed biomarker assays to detect panels of phosphorylated apoptotic proteins using patterned arrays on Meso Scale Discovery's MULTI-SPOT™ plates. Here we show multiplex detection of the following phosphoproteins: Akt, GSK-3α, Bad, and p53. Whole cell lysates are incubated on plates pre-coated with antibodies immobilized on four spatially distinct electrodes in a single well. The addition of detection antibodies, labeled with an electrochemiluminescent label, completes this sandwich immunoassay. Multiple proteins can be detected simultaneously in the same well with less than 0.05% optical cross-talk. Additionally, we also present an Akt assay in which total and phosphorylated Akt are detected in the same well. Both assays afford fast, simple protocols in which the results obtained from treated and untreated cells agree with those obtained by traditional western blot analysis.

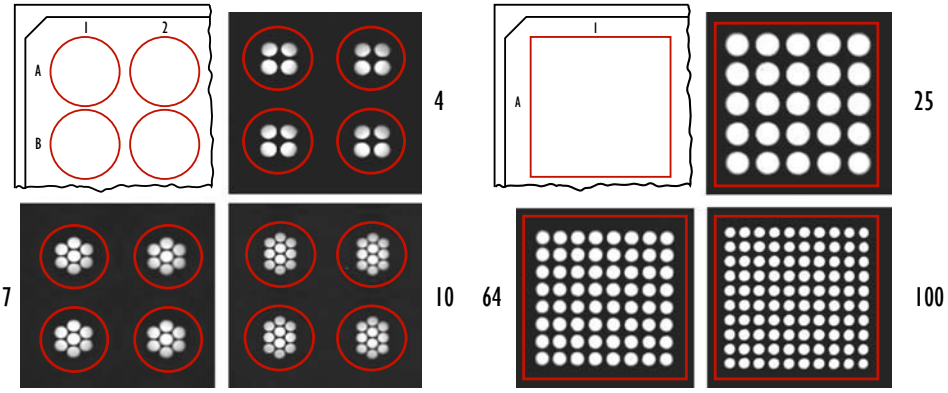
❷ Meso Scale Discovery (MSD) MULTI-SPOT™ Plates

SECTOR™ Imager 6000

Screen printing affords easy patterning.

Microfluidics allows "addressing" to immobilize distinct species on each electrode.

SECTOR™ Imager 6000 reader results ≤ 0.05% optical cross-talk.



96 wells: 4, 7, and 10 spots/well

24 wells: 25, 64, and 100 spots/well

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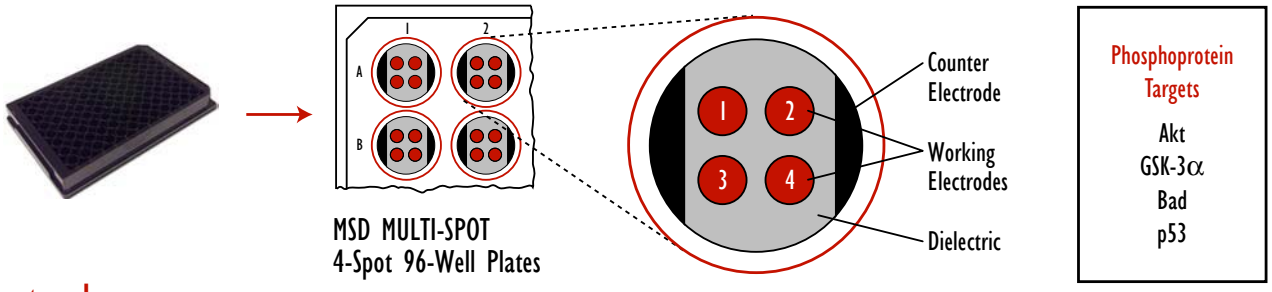
❸ Apoptosis Phosphoprotein Assay Format



Protocol

1. MSD MULTI-SPOT 4 Spot 96-Well plates precoated with capture antibodies are blocked with 3% BSA in TBS buffer (150mM NaCl, 50mM Tris-HCl pH7.5), 50µL per well, 2h. Wash with TBS
3. Cell lysates are incubated in the assay plate for 1h with shaking, 25µL per well. Lysate diluent: TBS buffer with fresh phosphatase inhibitor cocktails I and II, and a protease inhibitor cocktail. Wash with TBS
4. Antibodies labeled with MSD SULFO-TAG™ in TBS buffer with 1% MSD Blocker A are pre-mixed and incubated in the assay plate for 1h with shaking, 25µL per well. Wash with TBS
5. MSD Read Buffer T (1X), 150µL per well, followed by plate analysis on an MSD SECTOR Imager instrument.

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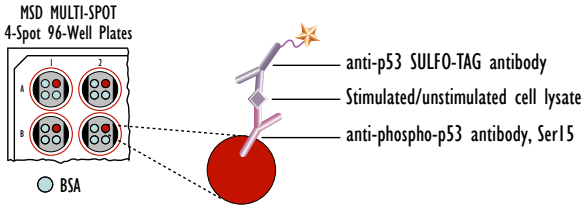
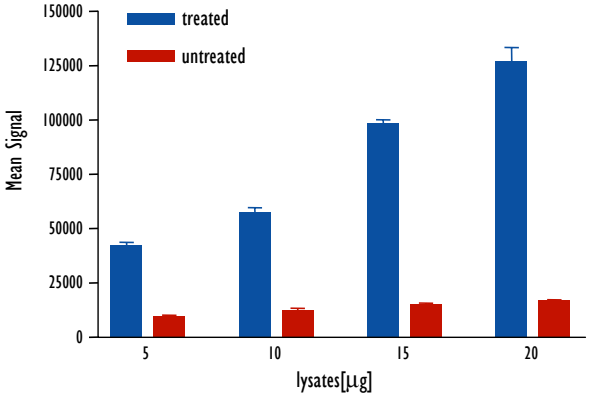
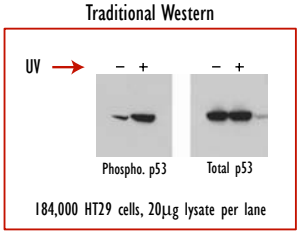
❹ Detection of Phosphorylated p53 in Whole Cell Lysates







Media was removed from logarithmically growing HT29 cells, followed by UV irradiation at 40mJ/cm². Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with anti-phospho-p53 antibody on one of the four spatially distinct electrodes per well. BSA was coated onto the remaining three electrodes in each well. Phosphorylated p53 was detected with 10nM anti-total-p53 antibody labeled with MSD SULFO-TAG reagent.

Lysate (µg)	p53 lysates (treated cells)			p53 lysates (untreated cells)			S-B	S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		
5	42,012	1,661	4	9,708	420	4	32,304	4.3
10	57,669	2,065	4	12,315	955	8	45,355	4.7
15	98,496	1,748	2	15,168	516	3	83,328	6.5
20	126,883	6,581	5	17,115	247	1	109,768	7.4

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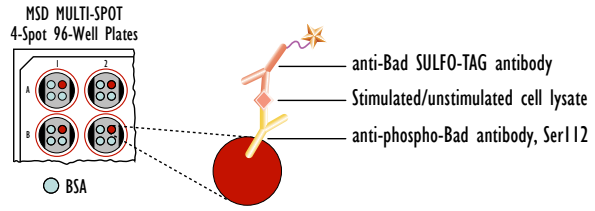
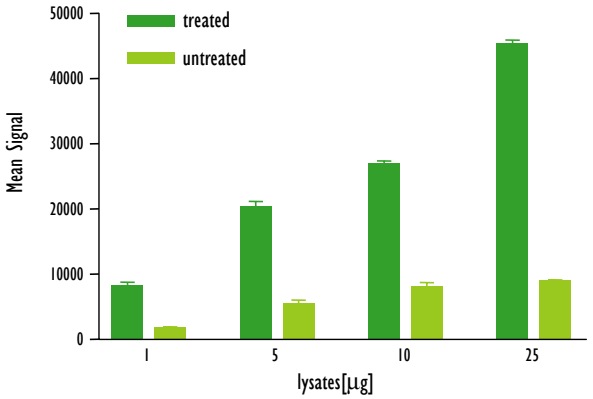
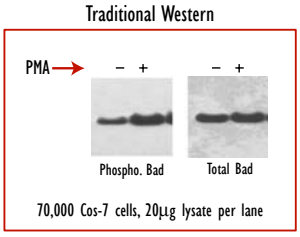
❺ Detection of Phosphorylated Bad in Whole Cell Lysates





Lysate (µg)	Bad lysates (treated cells)			Bad lysates (untreated cells)			S-B	S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		
1	8,417	378	4	1,894	37	2	6,523	4.4
5	20,459	819	4	5,603	490	9	14,856	3.7
10	27,081	351	1	8,232	587	7	18,849	3.3
20	45,531	496	1	9,202	18	0	36,329	4.9



Logarithmically growing Cos-7 cells were serum-starved overnight, followed by treatment with PMA for 1h. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with anti-phospho-Bad antibody on one of the four spatially distinct electrodes per well. BSA was coated onto the remaining three electrodes in each well. Phosphorylated Bad was detected with 10nM anti-total-Bad antibody labeled with MSD SULFO-TAG reagent.

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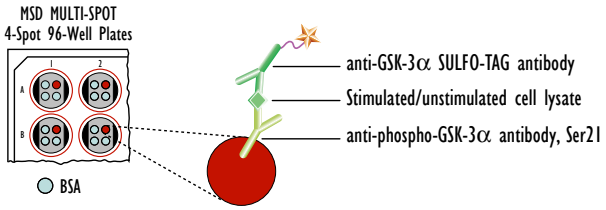
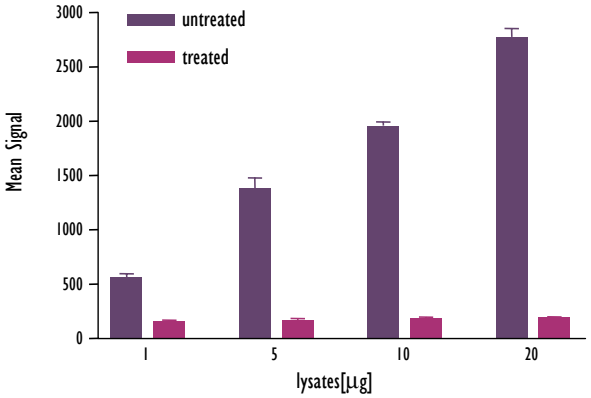
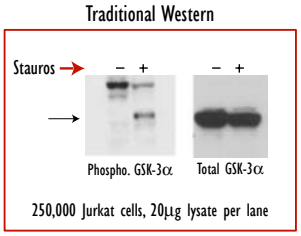
6 Detection of Phosphorylated GSK-3α in Whole Cell Lysates





Lysate (µg)	GSK lysates (untreated cells)			GSK lysates (treated cells)			S-B	S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		
1	565	33	6	163	4	2	402	3.5
5	1,387	90	6	175	11	6	1212	7.9
10	1,960	36	2	191	3	1	1769	10.3
20	2,771	80	3	198	2	1	2573	14.0



Logarithmically growing Jurkat cells were treated with staurosporine for 4h. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with anti-phospho-GSK-3α antibody on one of the four spatially distinct electrodes per well. BSA was coated onto the remaining three electrodes in each well. Phosphorylated GSK-3α was detected with 10nM anti-total-GSK-3α antibody labeled with MSD SULFO-TAG reagent.

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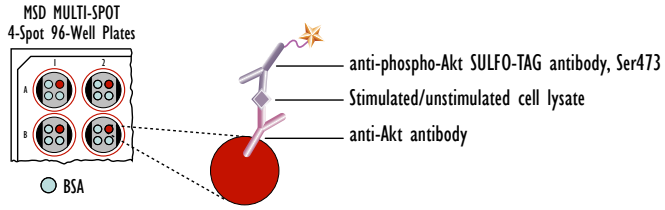
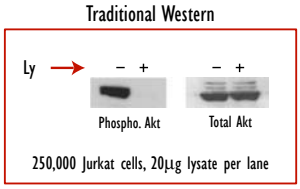
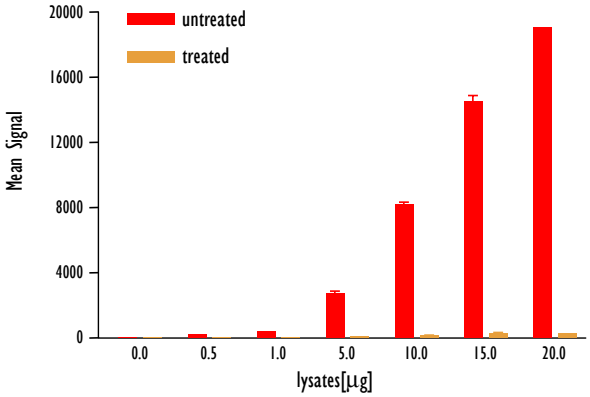
7 Detection of Phosphorylated Akt in Whole Cell Lysates







Logarithmically growing Jurkat cells were treated with Ly inhibitor for 1h. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with anti-pan-Akt antibody on one of the four spatially distinct electrodes per well. BSA was coated onto the remaining three electrodes in each well. Phosphorylated Akt was detected with 10nM anti-phospho-Akt antibody labeled with MSD SULFO-TAG reagent.

Lysate (µg)	Akt lysates (untreated cells)			Akt lysates (treated cells)			S-B	S/B
	Ave ECL	Std.Dev.	%CV	Ave ECL	Std.Dev.	%CV		
0	45	5	11	37	7	19	8	1.2
0.5	200	23	12	39	1	4	161	5.1
1	393	6	1	64	6	9	329	6.1
5	2,738	134	5	127	4	3	2,611	21.6
10	8,196	145	2	169	11	6	8,027	48.6
15	14,485	406	3	261	25	10	14,224	55.5
20	19,034	7	0	327	6	2	18,708	58.3

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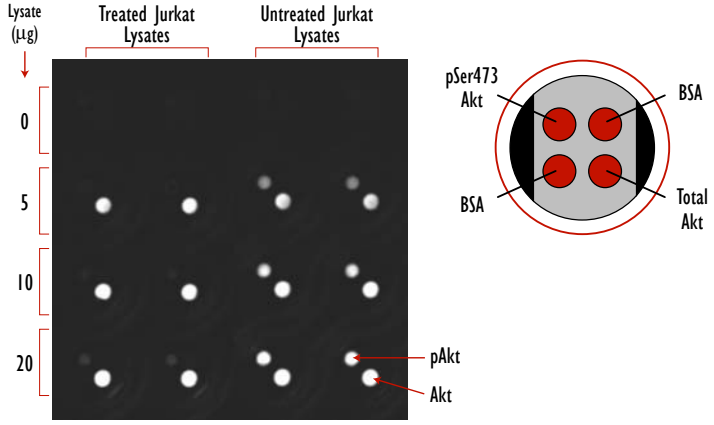
8 Multiplex Akt Assay: Detection of Phosphorylated and Total Akt in the Same Well



Logarithmically growing Jurkat cells were treated with Ly inhibitor for 1h. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with anti-phospho-Akt antibody and anti-total-Akt antibody coated on spatially distinct electrodes in the same well. BSA was coated onto the remaining two electrodes in each well. Phosphorylated and total Akt were detected with 10nM anti-total-Akt antibody labeled with MSD SULFO-TAG reagent. A titration of Jurkat cell lysates shows detection of increasing phosphorylated Akt while untreated/treated for total Akt remains constant.

Phospho-Akt

Lysate (µg)	p-Akt lysates (untreated cells)			p-Akt lysates (treated cells)			S-B	S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		
0	292	27	9	322	44	14	-30	0.9
5	6,592	445	7	952	11	1	5,640	6.9
10	12,832	49	0	1,356	74	5	11,476	9.5
20	21,964	236	1	2,056	124	6	19,878	10.5

Total-Akt

Lysate (µg)	p-Akt lysates (untreated cells)			p-Akt lysates (treated cells)			Untreated/Treated
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV	
0	154	20	13	131	1	1	1.2
5	17,347	854	5	22,577	3,340	15	0.8
10	26,362	151	1	36,862	956	3	0.7
20	41,392	2,100	5	54,564	1,625	3	0.8

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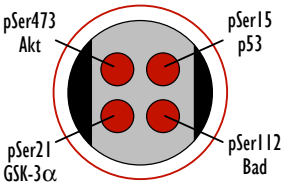
❾ Multiplex Apoptosis Panel: Detection of FOUR Phosphoproteins in the Same Well

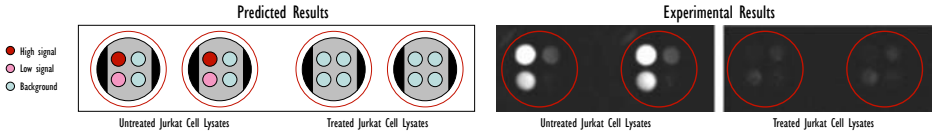
Whole cell lysates were added separately to MSD MULTI-SPOT 96-Well 4-spot plates pre-coated with anti-Akt, anti-Gsk-3α, anti-p53, and anti-Bad antibodies immobilized on four spatially distinct electrodes in a single well. Phosphorylated proteins were detected with reporter antibodies labeled with MSD SULFO-TAG reagent.



Reporter antibodies:
10nM anti-phospho-Akt-SULFO-TAG antibody,
10nM anti-Bad-SULFO-TAG antibody,
10nM anti-GSK-3α-SULFO-TAG antibody,
and 5nM anti-p53-SULFO-TAG antibody

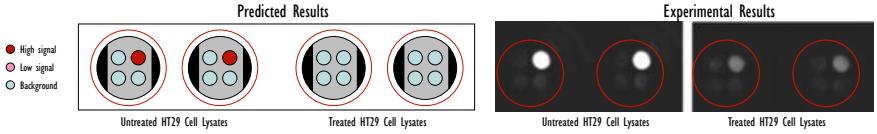
All four capture antibodies and detection antibodies in the well, Jurkat cell lysates only

Jurkat Lysate (µg)	Akt positive			Akt Negative			S/B	p53- positive			p53-negative			S/B	Gsk-positive			Gsk-negative			S/B	Bad-positive			Bad-negative			S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV	
0	714	4	0	706	38	5	1.0	1,028	11	1	946	33	4	1.1	1,333	66	5	1,257	33	3	1.1	1,466	68	5	1,467	48	3	1.0
10	7,210	298	4	544	28	5	13.3	592	29	5	627	16	2	0.9	4,226	115	3	891	16	2	4.7	1,046	208	20	847	6	1	1.2
20	18,423	363	2	726	42	6	25.4	575	20	3	601	16	3	1.0	6,042	514	9	873	16	2	6.9	1,522	11	1	834	12	1	1.8



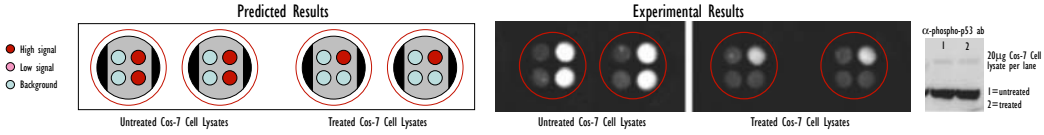
All four capture antibodies and detection antibodies in the well, HT29 cell lysates only

HT29 Lysate (µg)	Akt positive			Akt Negative			S/B	p53- positive			p53-negative			S/B	Gsk-positive			Gsk-negative			S/B	Bad-positive			Bad-negative			S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV	
0	418	89	21	418	89	21	1.0	263	47	18	263	47	18	1.0	343	78	23	343	47	14	1.0	361	42	12	361	42	12	1.0
10	1,545	78	5	1,849	99	5	0.8	16,154	5,720	35	4,403	1,029	23	3.7	1,489	172	12	1,596	1,029	64	0.9	1,749	118	7	1,707	100	6	1.0
20	1,731	11	1	1,984	24	1	0.9	27,759	7,845	28	5,487	1,066	19	5.1	1,616	243	15	1,569	1,066	68	1.0	2,121	117	6	1,759	168	10	1.2



All four capture antibodies and detection antibodies in the well, Cos-7 cell lysates only

Cos-7 Lysate (µg)	Akt positive			Akt Negative			S/B	p53- positive			p53-negative			S/B	Gsk-positive			Gsk-negative			S/B	Bad-positive			Bad-negative			S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV	
0	456	63	14	456	63	14	1.0	313	65	21	313	65	21	1.0	323	65	20	323	65	20	1.0	369	4	1	369	4	1	1.0
10	1,260	102	8	1,225	93	8	1.0	5,534	953	17	4,764	653	14	1.2	1,591	141	9	1,597	653	41	1.0	6,399	58	1	2,667	8	0	2.4
20	1,381	86	6	1,393	126	9	1.0	8,060	1,860	23	7,669	1,027	13	1.1	1,815	9	0	1,969	1,027	52	0.9	8,765	274	3	3,626	172	5	2.4



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⑩ Conclusions

- A panel of multiplex apoptotic phosphoprotein assays was developed to detect phospho-Akt, phospho-GSK-3α, phospho-p53 and phospho-Bad in whole cell lysates using MSD's MULTI-SPOT 4-Spot plates.
- Immuno-detection of phosphorylated Akt, GSK-3α, p53, and Bad was shown in individual assays in which a single antibody was immobilized in a well, as well as multiplexed with antibodies specific for each of the four targets immobilized within a single well.
- Detection of total and phosphorylated Akt in a single well was demonstrated with pan-Akt and phospho-specific Akt antibodies immobilized on diagonally opposed electrodes in a 4 Spot plate.
- Protocols for MSD multiplex assays are fast, simple and boast the sensitivity and specificity observed in traditional western blot analysis with whole cell lysates.