In vivo phosphorylation of key apoptotic and cell survival markers: simultaneous detection of phosphoproteins in a highly specific multiplex assay format

Paula Denney Eason, Bruk G. Leta,
Jenny T. Ly, Laura K. Schaefer,
Shayla R. Workman, Paul J. Goodwin,
Nisar Pampori, Robert M. Umek
and Jacob N. Wohlstadter

Meso Scale Discovery™
A division of Meso Scale Diagnostics, LLC.

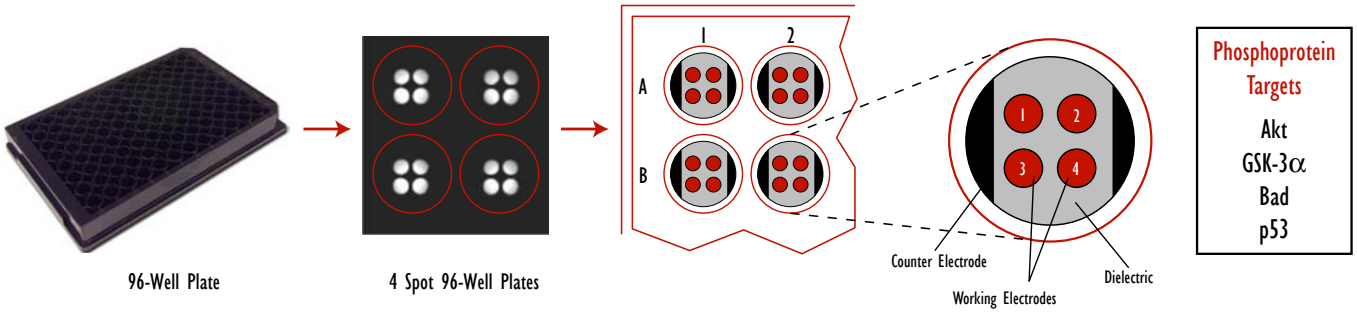
9238 Gaither Road, Gaithersburg, MD 20877
Phone: 240.631.2522 Fax: 240.632.2219
www.meso-scale.com

In vivo phosphorylation of key apoptotic and cell survival markers: simultaneous detection of phosphoproteins in a highly specific multiplex assay format

❶ Abstract

Apoptosis, or programmed cell death, plays a critical role in the control of disease and is the target of current drug discovery efforts to improve the efficacy of cancer therapeutics. Pro-survival pathways are similarly important in maintaining normal cell function where defects also lead to disease. Apoptosis and cell survival are both regulated through a variety of pathways acting in parallel that utilize phosphorylation as a key regulatory mechanism. However, limited approaches are available for simultaneously measuring key phosphorylated intermediaries in both of these pathways in a high throughput manner. Here we demonstrate the ability to detect simultaneously panels of the phosphorylated proteins Akt, GSK-3α, Bad, and p53 in a sensitive, quantifiable assay format. Additionally, the levels of phosphorylated protein can be compared to their total level; we demonstrate this measurement with the quantitation of phosphorylated and total Akt in Jurkat whole cell lysates 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. We think these measurements provide new tools towards the comprehensive understanding of signaling pathways in diseased and normal tissue.

❷ Apoptosis Phosphoprotein Assay Format



96-Well Plate

4 Spot 96-Well Plates

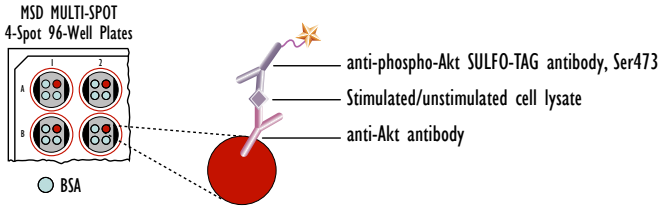
Protocol

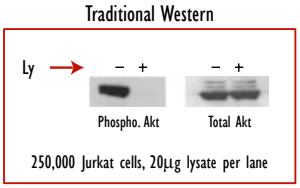
1. MSD MULTI-SPOT™ 4 Spot 96-Well plates precoated with capture antibodies are blocked with 3% BSA in TBS buffer (150mM NaCl, 50mM Tris-HCl pH7.5), 50µL per well, 2h. Wash with TBS
2. Cell lysates are incubated in the assay plate for 1h with shaking, 25µL per well. Lysate diluent: TBS buffer with fresh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

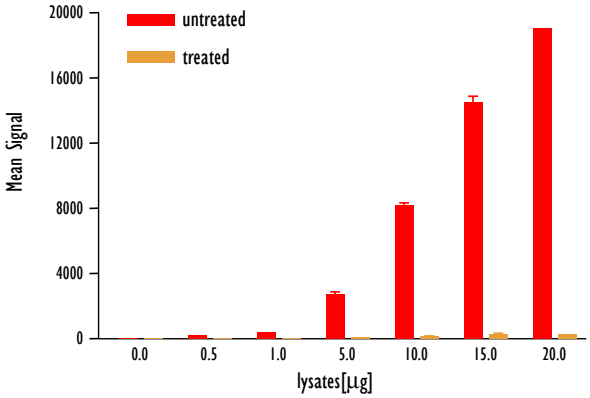
Meso Scale Discovery™
A division of Meso Scale Diagnostics, LLC.

In vivo phosphorylation of key apoptotic and cell survival markers: simultaneous detection of phosphoproteins in a highly specific multiplex assay format

❸ Detection of Phosphorylated Akt in Whole Cell Lysates





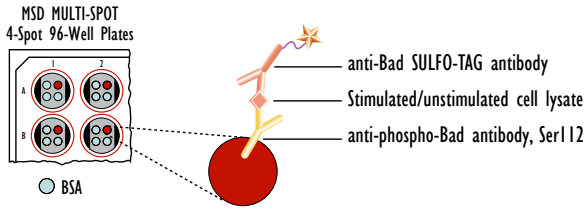


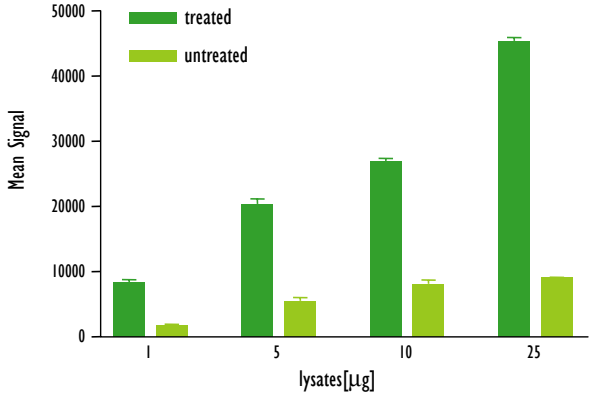
Logarithmically growing Jurkat cells were treated with Ly inhibitor for 1h. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with anti-pan-Akt antibody on one of the four spatially distinct electrodes per well. BSA was coated onto the remaining three electrodes in each well.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

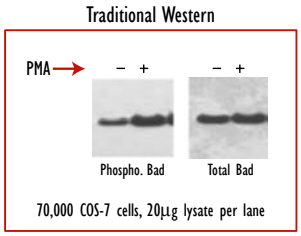
In vivo phosphorylation of key apoptotic and cell survival markers: simultaneous detection of phosphoproteins in a highly specific multiplex assay format

4 Detection of Phosphorylated Bad in Whole Cell Lysates





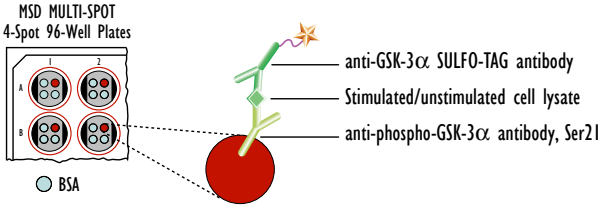
Lysate (µg)	Bad lysates (treated cells)			Bad lysates (untreated cells)			S-B	S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		
1	8,417	378	4	1,894	37	2	6,523	4.4
5	20,459	819	4	5,603	490	9	14,856	3.7
10	27,081	351	1	8,232	587	7	18,849	3.3
20	45,531	496	1	9,202	18	0	36,329	4.9

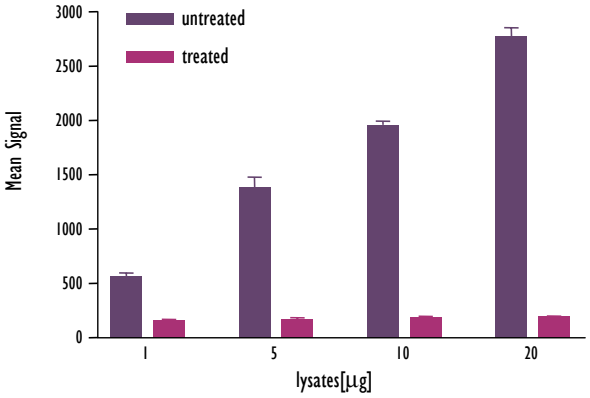


Logarithmically growing COS-7 cells were serum-starved overnight, followed by treatment with PMA for 1h. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with anti-phospho-Bad antibody on one of the four spatially distinct electrodes per well. BSA was coated onto the remaining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

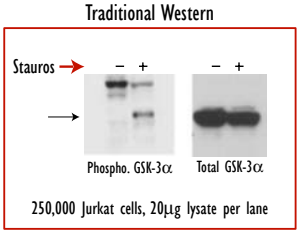
In vivo phosphorylation of key apoptotic and cell survival markers: simultaneous detection of phosphoproteins in a highly specific multiplex assay format

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





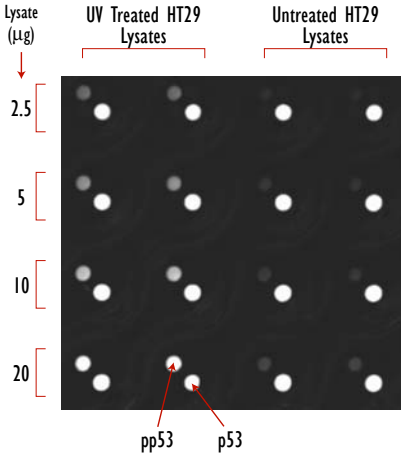
Lysate (µg)	GSK lysates (untreated cells)			GSK lysates (treated cells)			S-B	S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		
1	565	33	6	163	4	2	402	3.5
5	1,387	90	6	175	11	6	1212	7.9
10	1,960	36	2	191	3	1	1769	10.3
20	2,771	80	3	198	2	1	2573	14.0

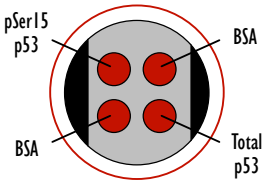


Logarithmically growing Jurkat cells were treated with staurosporine for 4h. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with anti-phospho-GSK-3α antibody on one of the four spatially distinct electrodes per well. BSA was coated onto the remaining three electrodes in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

In vivo phosphorylation of key apoptotic and cell survival markers: simultaneous detection of phosphoproteins in a highly specific multiplex assay format

6 Multiplex p53 Assay: Detection of Phosphorylated and Total p53 in the Same Well





Media was removed from logarithmically growing HT29 cells, followed by UV irradiation at 40mJ/cm^2. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well High Bind plates coated with anti-phospho-p53 antibody and anti-total-p53 antibody coated on spatially distinct electrodes in the same well. BSA was coated onto the remaining two electrodes in each well. Phosphorylated and total p53 were detected with 5nM anti-total-p53 antibody labeled with MSD SULFO-TAG reagent. A titration of HT29 cell lysates shows detection of increasing phosphorylated p53 while the ratio of treated/untreated total p53 remains constant.

Phospho-p53

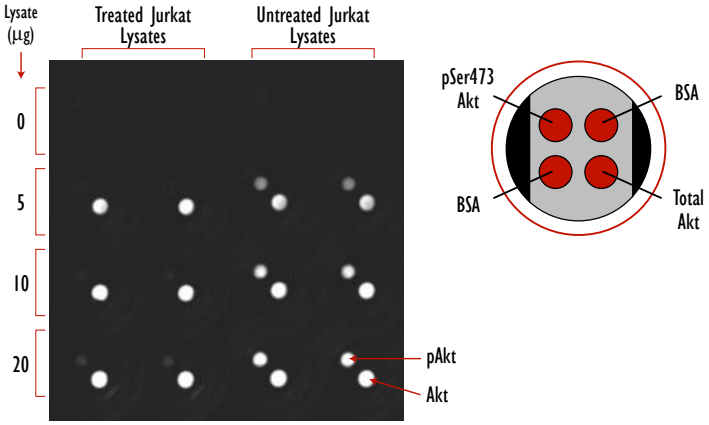
Lysate (µg)	p-p53 (treated cells)			p-p53 (untreated cells)			S/B
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV	
0	141	4	3	165	5	3	0.9
0.3125	8,506	132	2	7,316	612	8	1.2
0.625	14,904	474	3	10,418	1,095	11	1.4
1.25	20,206	482	2	10,422	1,111	11	1.9
2.5	24,992	2,850	11	8,035	1,323	16	3.1
5	40,285	611	2	8,585	48	1	4.7
10	53,398	2,809	5	9,011	127	1	5.9
20	81,312	462	1	11,525	531	5	7.1

Total p53

Lysate (µg)	p53 (treated cells)			p53 (untreated cells)			Treated/ Untreated
	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV	
0	695	187	27	588	98	17	1.2
0.3125	24,204	1,877	8	24,369	4,078	17	1.0
0.625	46,860	986	2	50,236	10,960	22	0.9
1.25	76,646	3,594	5	79,023	29,241	37	1.0
2.5	120,236	8,567	7	129,232	25,356	20	0.9
5	147,757	18,393	12	197,627	14,181	1	0.7
10	167,174	4,006	2	252,273	9,170	8	0.7
20	257,884	66,759	26	316,840	60,796	19	0.8

In vivo phosphorylation of key apoptotic and cell survival markers: simultaneous detection of phosphoproteins in a highly specific multiplex assay format

❼ 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

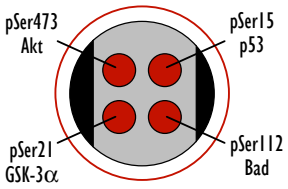
Total-Akt

Lysate (µg)	Akt lysates (untreated cells)			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

In vivo phosphorylation of key apoptotic and cell survival markers: simultaneous detection of phosphoproteins in a highly specific multiplex assay format

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

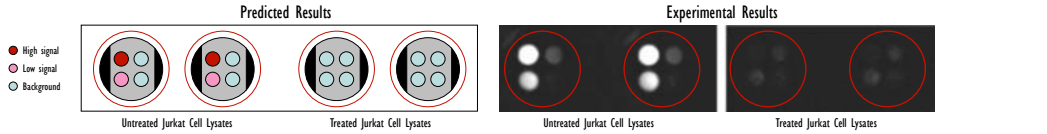
Whole cell lysates were added separately to MSD MULTI-SPOT 96-Well 4 Spot plates pre-coated with anti-Akt, anti-Gsk-3α, anti-p53, and anti-Bad antibodies immobilized on four spatially distinct electrodes in a single well. Phosphorylated proteins were detected with reporter antibodies labeled with MSD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

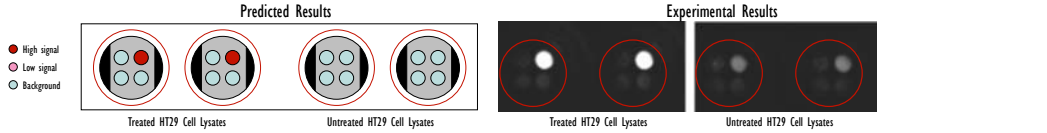
All four capture antibodies and detection antibodies in the well, Jurkat cell lysates only

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



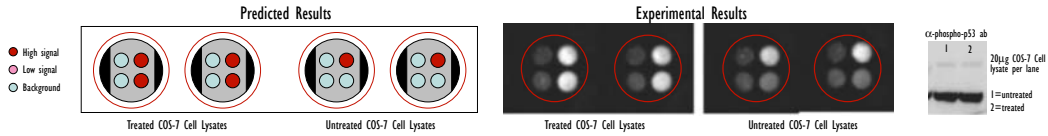
All four capture antibodies and detection antibodies in the well, HT29 cell lysates only

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



Results with Jurkat whole cell lysates show that only phosphorylated Akt and GSK-3α are detected. Phosphorylated p53 is detected with HT29 cell lysates, but not any of the other three markers. COS-7 cell lysates contain endogenous p53, shown in the western blot and confirmed in this multiplex assay. Therefore, we predict and have demonstrated here that the data and corresponding images show the same level of endogenous p53 for both treated and untreated COS-7 cell lysates, in addition to detection of phosphorylated Bad for treated cells. Individual measurements from each of the four spots remained highly analytical as regards sensitivity and precision and showed no spurious crosstalk between putative measurements, underscoring the utility of the method.

Meso Scale Discovery™
A division of Meso Scale Diagnostics, LLC.

In vivo phosphorylation of key apoptotic and cell survival markers: simultaneous detection of phosphoproteins in a highly specific multiplex assay format

9 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. The system allowed us to evaluate simultaneously several key regulatory proteins in the apoptosis and cell survival pathways. First, we demonstrated that the four phosphoproteins could be individually measured with very high specificity from select cell types. Besides detecting Akt from growing Jurkat cells, the key downstream partners to the Akt signaling pathway acting to prevent programmed cell death, phospho-Bad and phospho-GSK-3α were detected from PMA treated COS-7 cells and staurosporine treated Jurkat cells, respectively, with high signal to background ratios and excellent precision. As expected, when phospho-Akt was suppressed selectively by the PI3K specific LY294002 inhibitor, total Akt in these cells did not change as demonstrated by our duplex assay that simultaneously detected the two forms in a single well. Parallel to this, a duplex assay was developed to detect phospho and total forms of p53 from UV treated HT29 cells. The detection assays were further multiplexed to accommodate all four phosphoprotein targets in a single well. Protocols for MSD multiplex assays are fast, simple and boast the sensitivity and specificity observed in traditional western blot analysis with whole cell lysates. The specific detection of each protein in these multiplex panels presents a versatile analytical tool for determining the status of these proteins with high precision. These methods should be useful in elucidating mechanisms of apoptosis and, more generally, in checking and cross checking the status of various signaling pathways in the presence of stimuli.