Revolutionizing Signal Transduction Assays in Drug Discovery: MSD's Multiplex Phosphoprotein Assays with Cell Signaling Technology's Antibodies

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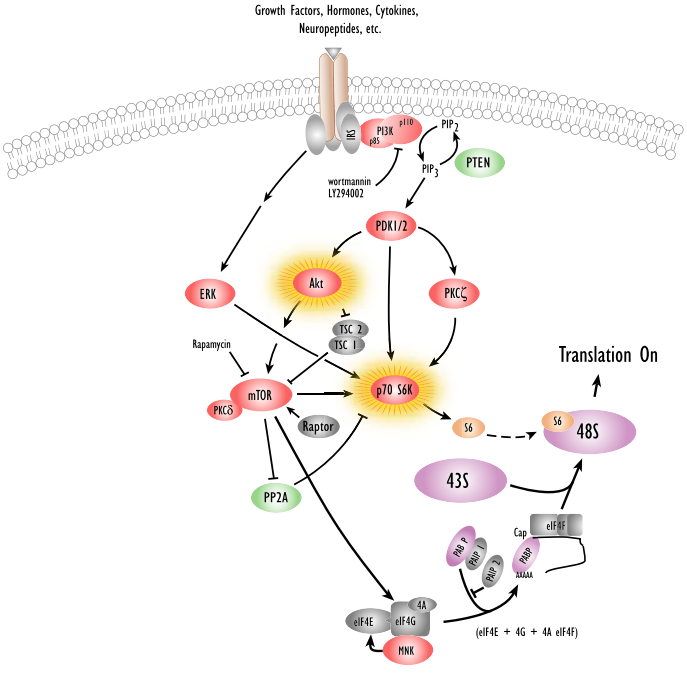
❶ Abstract

Meso Scale Discovery (MSD) has introduced a novel, high throughput platform for multiplex analysis of phosphoproteins. The platform utilizes arrays of anti-phosphoprotein antibodies that are immobilized at the bottom of microplates. In addition, detection antibodies that harbor a common reporter moiety are included in MSD's multiplex kits. The ability to combine any given set of antibodies in an array provides maximum flexibility. It is advantageous to combine antibodies that will report the phosphorylation status of various signal transducers known to be aligned in a pathway. In this manner, the point of action of a novel inhibitor can be characterized. These "pathway screens" are a powerful means of characterizing compounds in primary or secondary screens. Alternatively, targets from a pathway of interest can be combined with those in parallel pathways as a means of determining the specificity of a compound or series of compounds. MSD multiplex phosphoprotein detection kits feature antibodies from Cell Signaling Technology (CST). CST is recognized as a world leader in the development and manufacture of tools for signal transduction research. CST's anti-phosphoprotein antibodies are well-characterized with respect to their specificity and their performance in conventional technologies. Here, we demonstrate the performance of CST antibodies in MSD multiplex assays that are focused on known signal transduction pathways. The combination of the MSD platform and CST antibodies has the potential to revolutionize signal transduction research in drug discovery.

❷ Translational Control: Regulation of p70 S6 Kinase

Pathway Description: S6 kinase plays a critical role in translational regulation. Several stimuli, including growth factors and cytokines, regulate p70 S6 kinase by initiating a phosphorylation cascade involving the sequential activation of PI3K, PDK1/2, Akt/PKB and FRAP/mTOR kinase. FRAP/mTOR, together with an unidentified kinase, phosphorylates 4E-BP, leading to its dissociation from and activation of eIF4E. Phosphorylation of ribosomal protein S6 by p70 S6 kinase stimulates the translation of mRNAs with a 5' oligopyrimidine tract which typically encode components of the protein synthesis apparatus.



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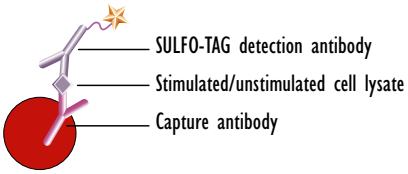
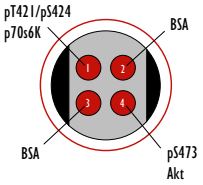
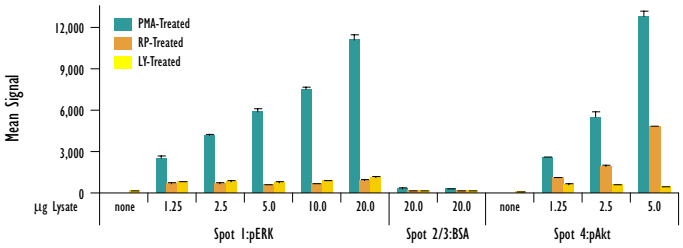
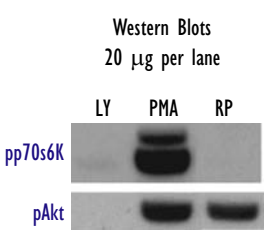
❷ Translational Control: Regulation of p70 S6 Kinase (continued)

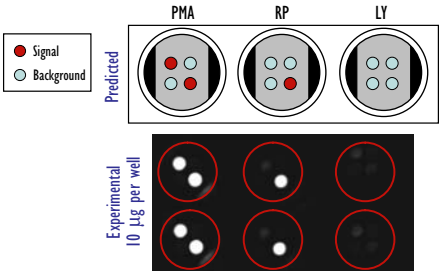
Multiplex Assay for Phospho-Akt and Phospho-p70s6K











Logarithmically growing Jurkat cells were treated with LY, Rapamycin, and 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.

Spot	Jurkat µg	PMA-Treated S1 Ave	PMA-Treated S1 %CV	RP-Treated S2 Ave	RP-Treated S2 %CV	LY-Treated B1 Ave	LY-Treated B1 %CV	S2/B1	S1/B1
1:p70s6K	1.25	2,573	5	699	5	814	3	0.9	3.2
1:p70s6K	2.5	4,157	3	669	11	847	2	0.8	4.9
1:p70s6K	5.0	5,855	5	591	6	782	5	0.8	7.5
1:p70s6K	10.0	7,517	2	665	3	879	2	0.8	8.6
1:p70s6K	20.0	11,091	4	885	6	1,141	2	0.8	9.7
2:BSA	20.0	303	12	166	9	162	4	1.0	1.9
2:BSA	20.0	282	4	163	5	161	2	1.0	1.7
3:pAkt	1.25	2,626	1	1,094	2	633	1	1.7	4.2
3:pAkt	2.5	5,531	7	1,975	4	613	2	3.2	9.0
3:pAkt	5.0	12,828	3	4,822	1	428	3	11.3	30.0
3:pAkt	10.0	26,572	3	9,907	5	336	5	29.5	79.2
3:pAkt	20.0	63,463	8	21,935	5	334	3	65.8	190.3

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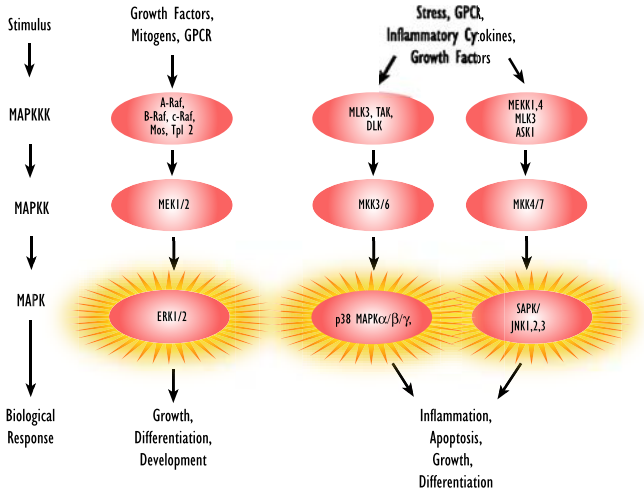
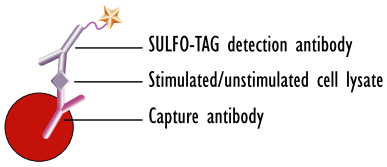
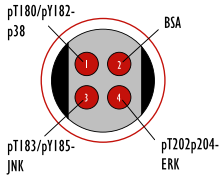
❸ MAPK Signal Cascades

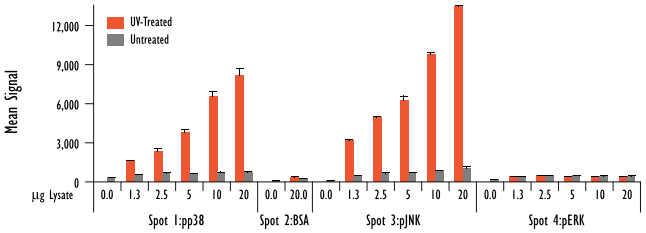
Pathway Description: Mitogen-activated protein kinases (MAPK) are a family of serine/threonine protein kinases widely conserved among eukaryotes and are involved in many cellular programs such as cell proliferation, cell differentiation, cell movement and cell death. MAPK signaling cascades are organized hierarchically into three-tiered modules. MAPKs are phosphorylated and activated by MAPK-kinases (MAPKKs), which in turn are phosphorylated and activated by MAPKK-kinases (MAPKKKs). The MAPKKK is in turn activated by interaction with a family of small GTPases and/or other protein kinases connecting the MAPK module to the cell surface receptor or external stimuli.

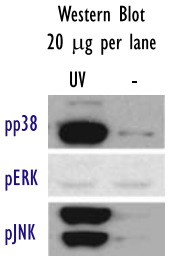


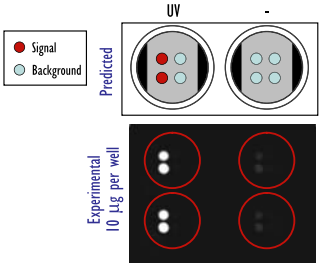
Multiplex Assays for MAP Kinases: Up-regulation of pp38 and pJNK











Logarithmically growing HEK293 cells were treated +/- UV. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well High Bind plates coated with capture antibody at three of the four spatially distinct electrodes per well. Phosphorylated proteins were detected with MSD SULFO-TAG detection antibodies.

	HEK293	UV-Treated		Untreated		S-B	S/B
Spot	µg	Ave	%CV	Ave	%CV		
1:pp38	none			300	5		
1:pp38	1.25	1,600	3	553	6	1,047	2.9
1:pp38	2.5	2,356	10	636	4	1,720	3.7
1:pp38	5.0	3,809	5	644	0	3,164	5.9
1:pp38	10.0	6,538	6	681	7	5,857	9.6
1:pp38	20.0	8,163	6	781	2	7,383	10.5
2:BSA	20.0	297	19	231	4	66	1.3
3:pJNK	none			113	17		
3:pJNK	1.25	3,180	3	481	2	2,700	6.6
3:pJNK	2.5	4,942	1	648	2	4,294	7.6
3:pJNK	5.0	6,246	5	680	3	5,566	9.2
3:pJNK	10.0	9,808	2	844	6	8,964	11.6
3:pJNK	20.0	13,437	1	1,064	9	12,373	12.6
4:pERK	20.0	437	4	461	4	-25	0.9

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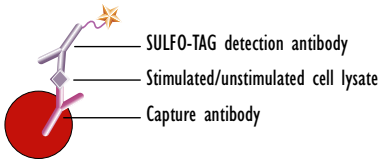
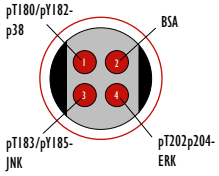
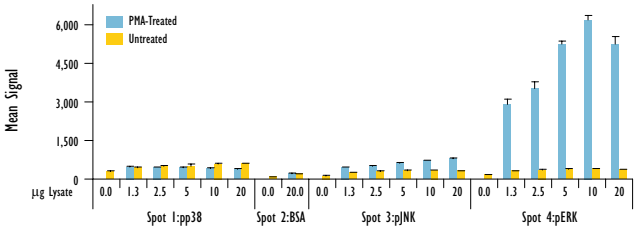
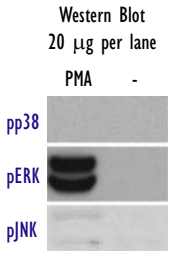
❸ MAPK Signal Cascades (continued)

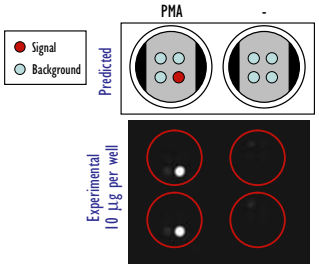
Multiplex Assays for MAP Kinases: Up-regulation of pERK











Spot	Jurkat µg	PMA-Treated Ave	PMA-Treated %CV	Untreated Ave	Untreated %CV	S-B	S/B
1:pp38	0.0			300	5		
1:pp38	20	386	6	605	4	-218	0.6
2:BSA	20	197	12	203	8	-7	1.0
3:pJNK	0.0			113	17		
3:pJNK	20	799	3	315	4	484	2.5
4:pERK	0.0			169	5		
4:pERK	1.3	2,903	7	319	1	2,584	9.1
4:pERK	2.5	3,516	8	363	8	3,154	9.7
4:pERK	5	5,274	2	368	12	4,907	14.3
4:pERK	10	6,200	3	411	3	5,790	15.1
4:pERK	20	5,274	5	384	1	4,890	13.7

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

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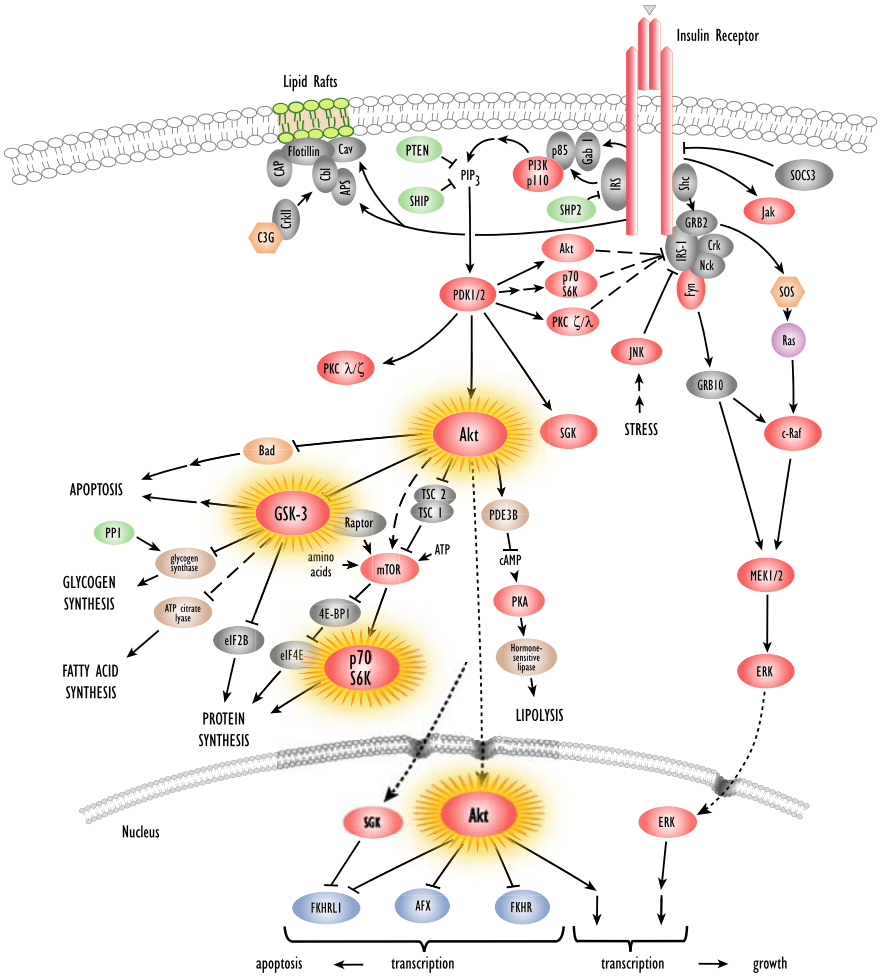
4 Insulin Receptor Signaling

IRS family of proteins. Tyrosine phosphorylated IRS then displays binding sites for numerous signaling partners. Among them, PI3K has a major role in insulin functions, mainly via the activation of the Akt/PKB. Activated Akt induces glycogen synthesis, through inhibition of GSK-3. Insulin signaling has also growth and mitogenic effects, which are mostly mediated by the Akt cascade, as well as by activation of the Ras/MAPK pathway. A negative feedback signal emanating from Akt/PKB, PKCz, p70 S6K and the MAPK cascades results in serine phosphorylation and inactivation of IRS signaling.



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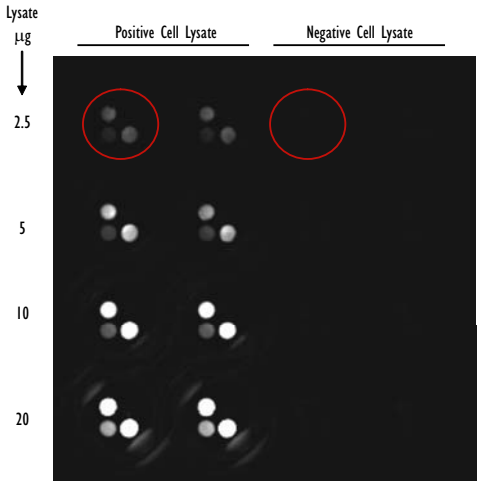
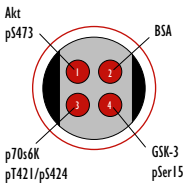
❹ Insulin Receptor Signaling (continued)

Multiplex Assay for pAkt, pGSK-3α & pp70s6K



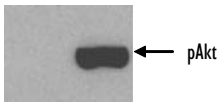


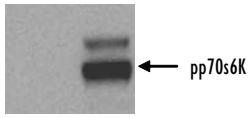
Lysates	pAkt-positive		pAkt-negative		S-B	S/B
mg	Ave	%CV	Ave	%CV		
0.0	123	5	122	1	1	
0.3	785	1	243	1	542	3.2
1.25	2,995	2	360	2	2,635	8.3
2.5	6,834	2	410	1	6,425	16.7
5	16,192	21	548	4	15,644	29.5
10	47,195	4	730	9	46,465	64.7
20	116,914	4	1,286	5	115,628	90.9

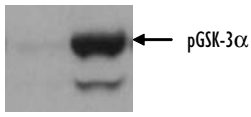
Lysates	pp70s6k-positive		pp70s6k-negative		S-B	S/B
µg	Ave	%CV	Ave	%CV		
0.0	136	22	105	6	32	
0.3	486	0	175	0	312	2.8
1.25	1,585	1	237	1	1,348	6.7
2.5	3,320	3	247	2	3,073	13.4
5	5,839	2	285	0	5,554	20.5
10	9,557	1	260	2	9,297	36.8
20	18,554	5	269	2	18,286	69.1

Lysates	pGSK-3α-positive		pGSK-3α-negative		S-B	S/B
µg	Ave	%CV	Ave	%CV		
0.0	125	11	120	2	5	
0.3	506	6	218	4	289	2.3
1.25	2,559	4	306	1	2,253	8.4
2.5	7,774	8	379	0	7,396	20.5
5	20,866	12	392	2	20,474	53.2
10	57,411	2	410	3	57,001	140.2
20	134,992	5	375	24	134,617	360.0

20 µg Lysate per lane







Logarithmically growing Jurkat cells were treated with PMA (positive) or LY/Staurosporine (negative). Whole cell lysates 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. Phosphorylated proteins were detected with MSD SULFO-TAG labeled detection antibodies.

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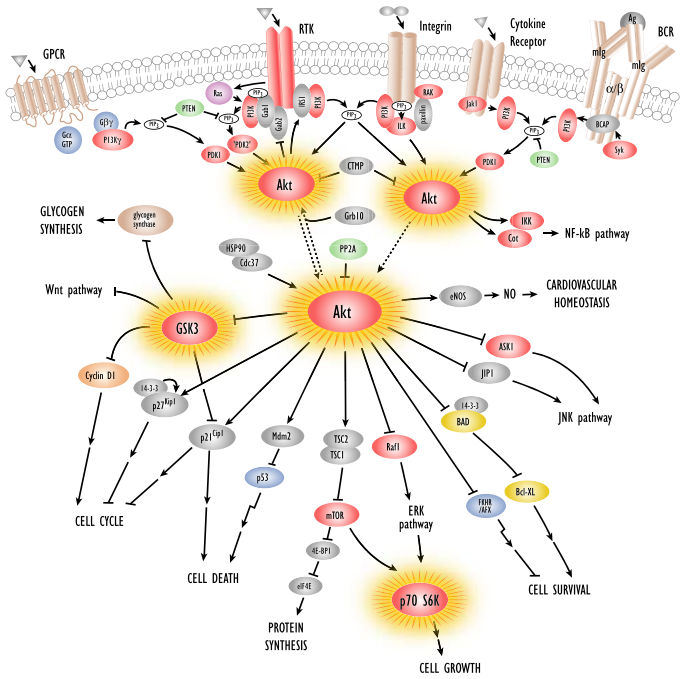
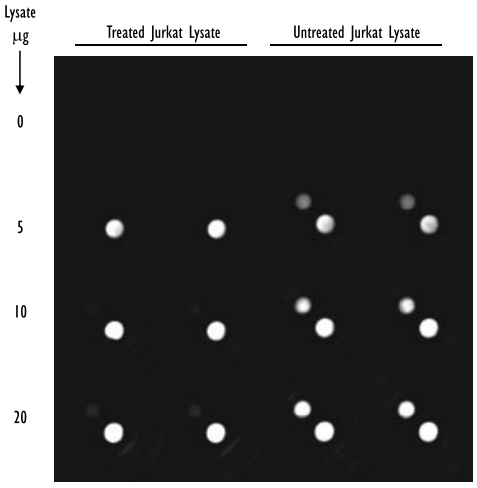
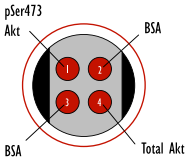
5 Akt/PKB Signaling Pathways

Pathway Description: Since its discovery as a proto-oncogene nearly ten years ago, the serine/threonine kinase Akt, also known as protein kinase B (PKB), has become a major focus of attention because of its critical regulatory role in diverse cellular processes, including cancer progression. The Akt cascade is activated by receptor tyrosine kinases, integrins, B- and T-cell receptors, cytokine receptors, G-protein coupled receptors, and other stimuli. The three Akt isoforms (Akt1, Akt2 and Akt3) thus mediate many of the downstream events regulated by PI3K. For instance, Akt is a major regulator of insulin signaling and glucose metabolism. Akt controls cell growth through its effects on the mTOR and p70 S6 kinase pathways, as well as the cell cycle and cell proliferation through its direct action on the CDK inhibitors, p21 and p27. Akt is also major mediator of cell survival by directly inhibiting different pro-apoptotic signals such as Bad and the Forkhead family of transcription factors. Akt signaling in endothelial cells also plays critical roles in the regulation of vascular homeostasis and angiogenesis. These findings have turned Akt/PKB into an important therapeutic target for the treatment of cancer, diabetes and stroke.



Multiplex Assay for Phosphorylated and Total Akt in the Same Well





Phospho Akt

Lysates µg	p-Akt Untreated			p-Akt Treated			S-B	S/B
	Ave	SD	%CV	Ave	SD	%CV		
0.0	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	2,086	124	6	19,878	10.5

Total Akt

Lysates µg	Total Akt Untreated			Total Akt Treated			Untreated/ Treated
	Ave	SD	%CV	Ave	SD	%CV	
0.0	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

Logarithmically growing Jurkat cells were treated with LY inhibitor for 1h. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well High Bind plates coated with α-phospho-Akt antibody and α-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. A titration of Jurkat cell lysates shows detection of increasing phosphorylated Akt while total Akt remains constant

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6 Conclusions

1. MSD provides kits for multiplex measurement of phosphoproteins
2. Arrays of anti-phosphoprotein antibodies empower "pathway screens" to characterize inhibitors
3. CST antibodies, well-established for conventional measurements, are brought to high throughput multiplexing on the MSD platform
4. The combination brings fast, accurate, multiplex measurement of phosphorylation status to signal transduction research in drug discovery