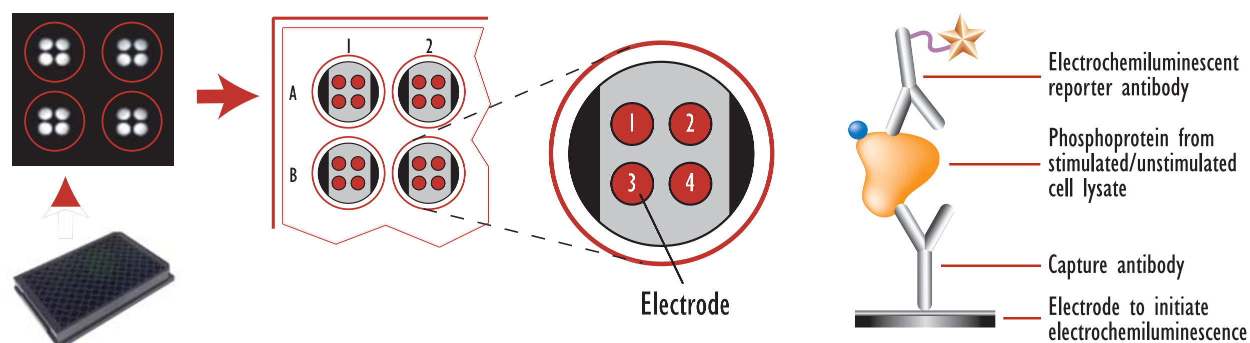
Multiplex Assays for Ubiquitous Signal Transduction Cascades: MAPK, Apoptosis and Akt/mTOR

Nisar Pampori, Jennifer Lewis, Bruk G. Leta, Laura K. Schaefer, Robert M. Umek, Paula Denney Eason and Jacob N. Wohlstadter

Protein phosphorylation is the major cellular mechanism used to regulate protein function including enzyme activity, protein-protein interactions, subcellular distribution, stability and degradation. These functions in turn control cell growth, death, differentiation, inflammation and apoptosis among other responses. The ability to assay phosphorylation status throughout signaling cascades is paramount to drug discovery and life science research. Ideally, multiplex assays allow simultaneous interrogation of multiple members of a signaling cascade. We have developed MULTI-ARRAY™ assays that simultaneously interrogate the phosphorylation state of key components of MAPK (ERK1/2, p38 and JNK), Apoptosis (Caspase-3, PARP, Bcl-2, BAD and p53) and Akt/mTOR (Akt, p70S6K, GSK-3α, GSK-3β and MEK1/2) signaling pathways. Furthermore, the percentage of protein that is phosphorylated at a given site can be estimated by simultaneously measuring the phosphorylated and total pools. The results closely mimic those seen in western blots. MULTI-ARRAY assays offer the advantages of multiplexing while being very sensitive and rapid. The combination of multiplexing and rapid protocols results in tremendous gains in productivity compared to conventional western blot and ELISA analyses.

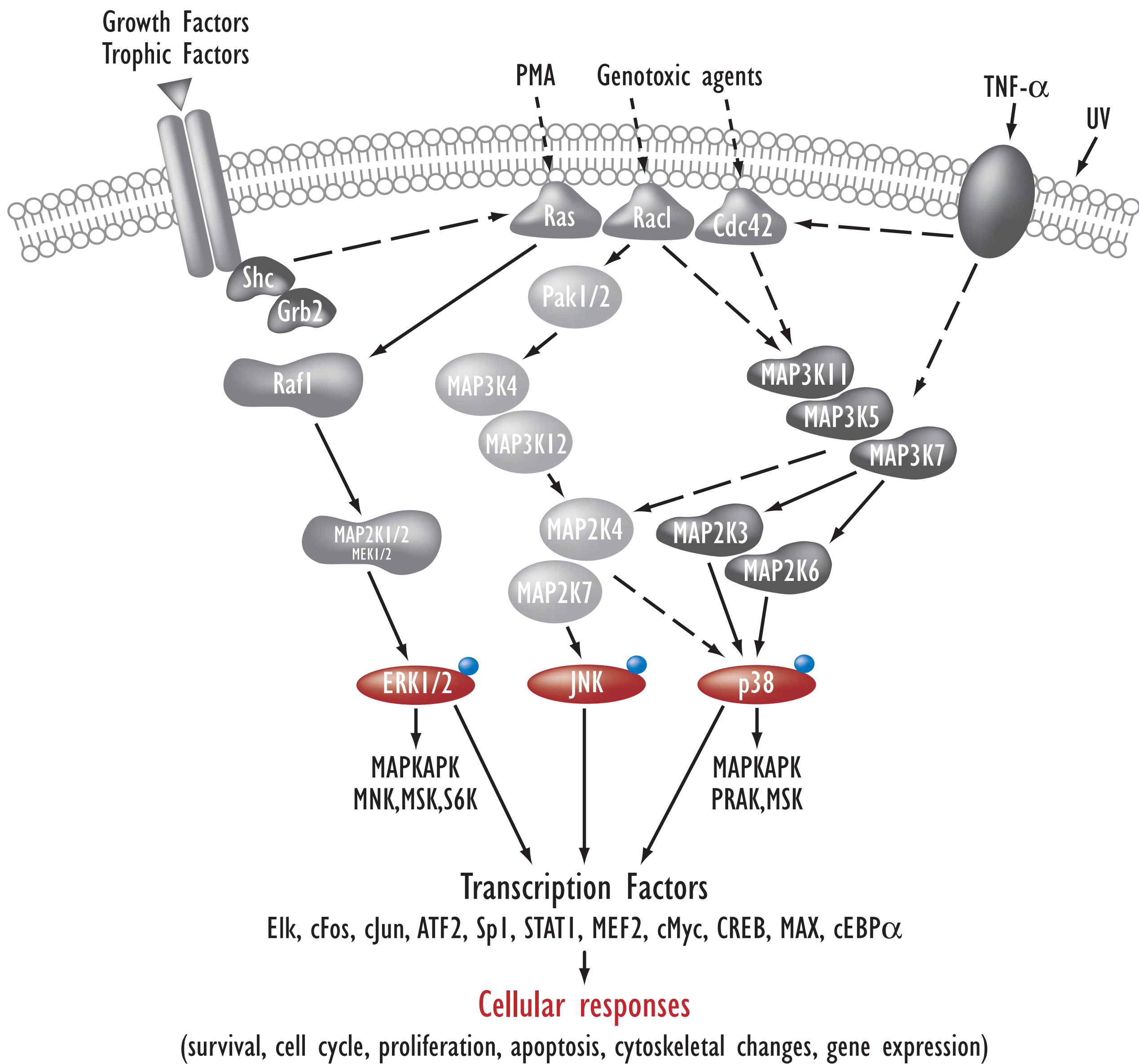
MSD MULTI-ARRAY Technology and MULTI-SPOT® Plates



1. MULTI-SPOT 4 Spot 96-Well Plates precoated with capture antibodies are blocked with 100 µL of MSD Blocker-A for 1 hr and washed with TBST.
2. 25 µL of cell lysates are added to the wells and incubated for 1-3 hr with shaking, followed by washing with TBST.
3. 25 µL MSD SULFO-TAG™ labeled antibodies (in antibody dilution buffer) are added to the wells and incubated for 1 hr with shaking, followed by washing with TBST.
4. 150 µL MSD Read Buffer T (with surfactant) are added to the wells and analyzed on the SECTOR™ 6000 instrument.

MSD

MAPK Signaling Pathway

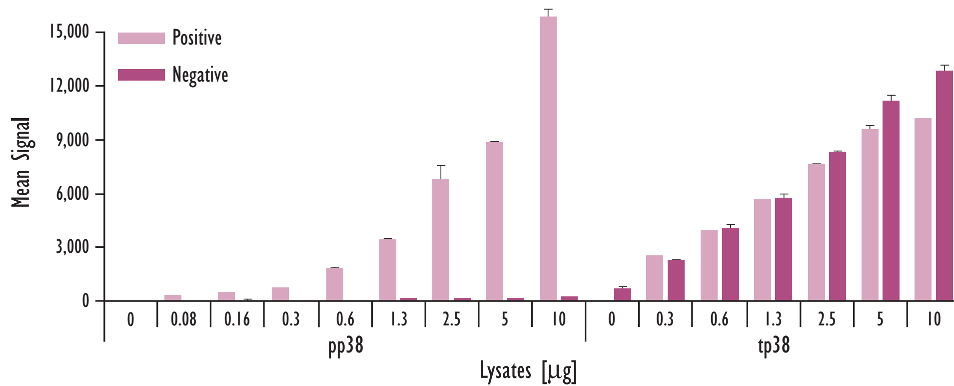


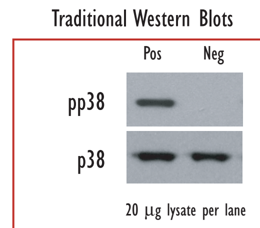
Phosphorylation modification

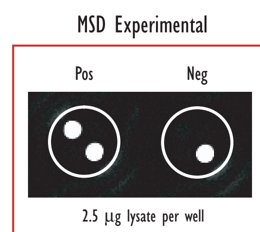
Targets analyzed in MSD multiplex formats

Multi-step pathway

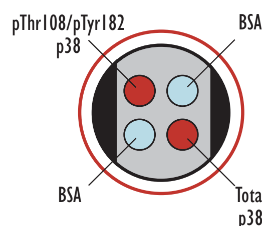
Multiplexed Phospho-p38 and Total p38 Assay in HEK293 Whole Cell Lysates





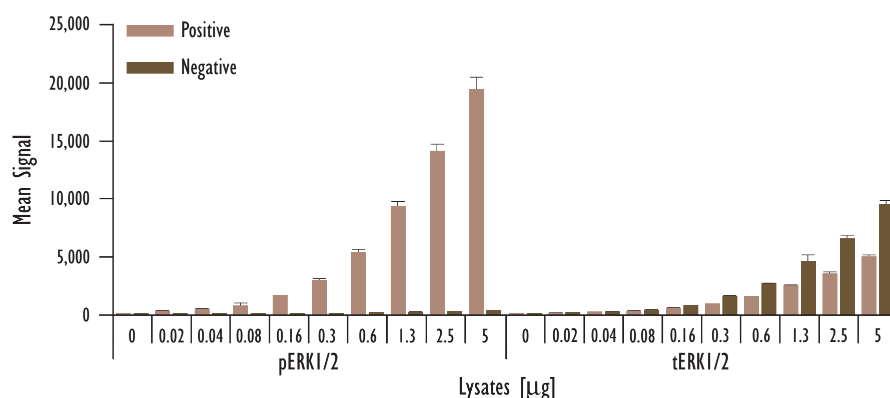


	Lysates (µg)	Positive		Negative		P/N
		Average	%CV	Average	%CV	
pp38	0			26	49	
	0.08	240	4	42	5	5.8
	0.16	433	7	43	18	10.2
	0.3	740	0	33	21	22.4
	0.6	1,818	5	38	4	47.8
	1.3	3,396	3	61	22	56.1
	2.5	6,822	11	84	6	81.7
	5	8,805	1	123	5	71.6
	10	15,850	3	197	8	80.7
tp38	0			660	18	
	0.3	2,464	0	2,251	3	1.1
	0.6	3,885	0	4,016	7	1.0
	1.3	5,630	0	5,702	4	1.0
	2.5	7,538	1	8,278	1	0.9
	5	9,558	3	11,165	3	0.9
	10	10,187	0	12,849	2	0.8

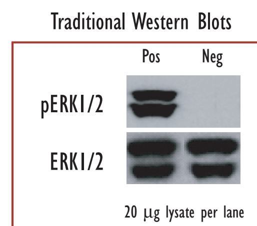


Logarithmically growing HEK293 cells were treated with UV + Calyculin A (positive) or rapamycin (negative). Whole cell lysates were added to MSD MULTI-SPOT 4-spot plates (pre-coated with capture antibody) and analyzed in a multiplexed sandwich assay.

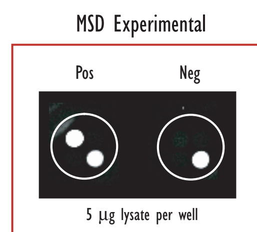
Multiplexed Phospho-ERK1/2 and Total ERK1/2 Assay in Jurkat Whole Cell Lysates



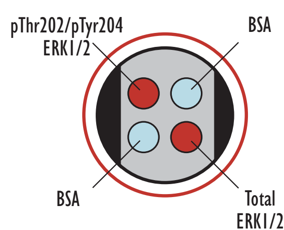
Traditional Western Blots



MSD Experimental

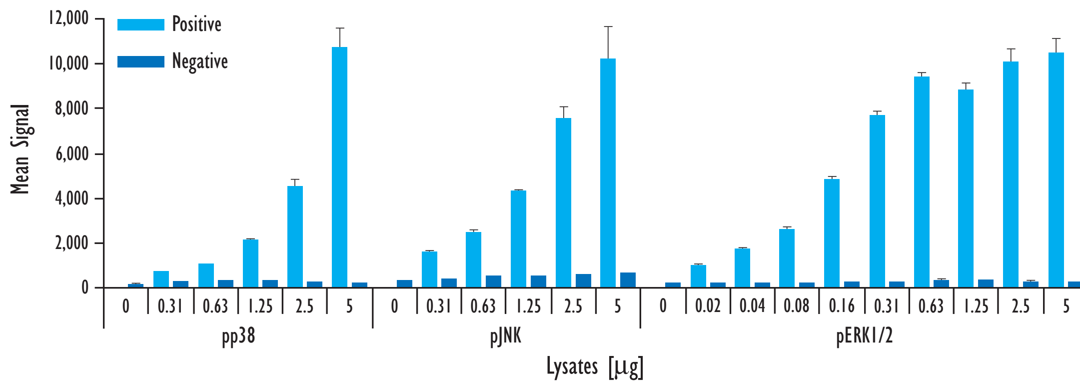


	Lysates (µg)	pERK1/2 Positive			pERK1/2 Negative			P/N
		Average	StdDev	%CV	Average	StdDev	%CV	
pERK1/2	0	154	16	10	142	10	7	
	0.02	343	21	6	154	16	10	2.2
	0.04	511	21	4	134	7	5	3.8
	0.08	849	191	22	145	7	5	5.9
	0.16	1,660	50	3	148	8	5	11.2
	0.3	2,979	170	6	181	8	4	16.4
	0.6	5,447	228	4	217	12	5	25.1
	1.3	9,324	438	5	267	18	7	34.9
	2.5	14,091	665	5	317	4	1	44.5
	5	19,386	1,110	6	385	15	4	50.4
tERK1/2	0	151	11	7	151	18	12	
	0.02	197	12	6	205	11	5	1
	0.04	238	6	3	281	12	4	0.8
	0.08	351	23	6	444	6	1	0.8
	0.16	581	30	5	812	29	4	0.7
	0.3	973	23	2	1,629	42	3	0.6
	0.6	1,625	19	1	2,661	55	2	0.6
	1.3	2,500	89	4	4,590	578	13	0.5
	2.5	3,580	133	4	6,588	316	5	0.5
	5	4,999	163	3	9,544	355	4	0.5

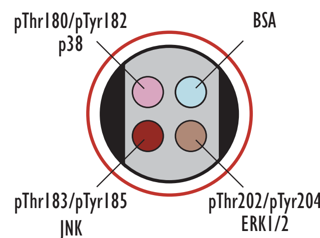


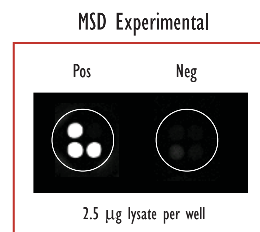
Logarithmically growing Jurkat cells were treated with PMA (positive) or LY294002 (negative). Whole cell lysates were added to MSD MULTI-SPOT 4-spot plates (pre-coated with capture antibody) and analyzed in a multiplexed sandwich assay.

Multiplexed MAPK Panel: Phospho-p38, Phospho-JNK and Phospho-ERK1/2 in HEK293 Whole Cell Lysates



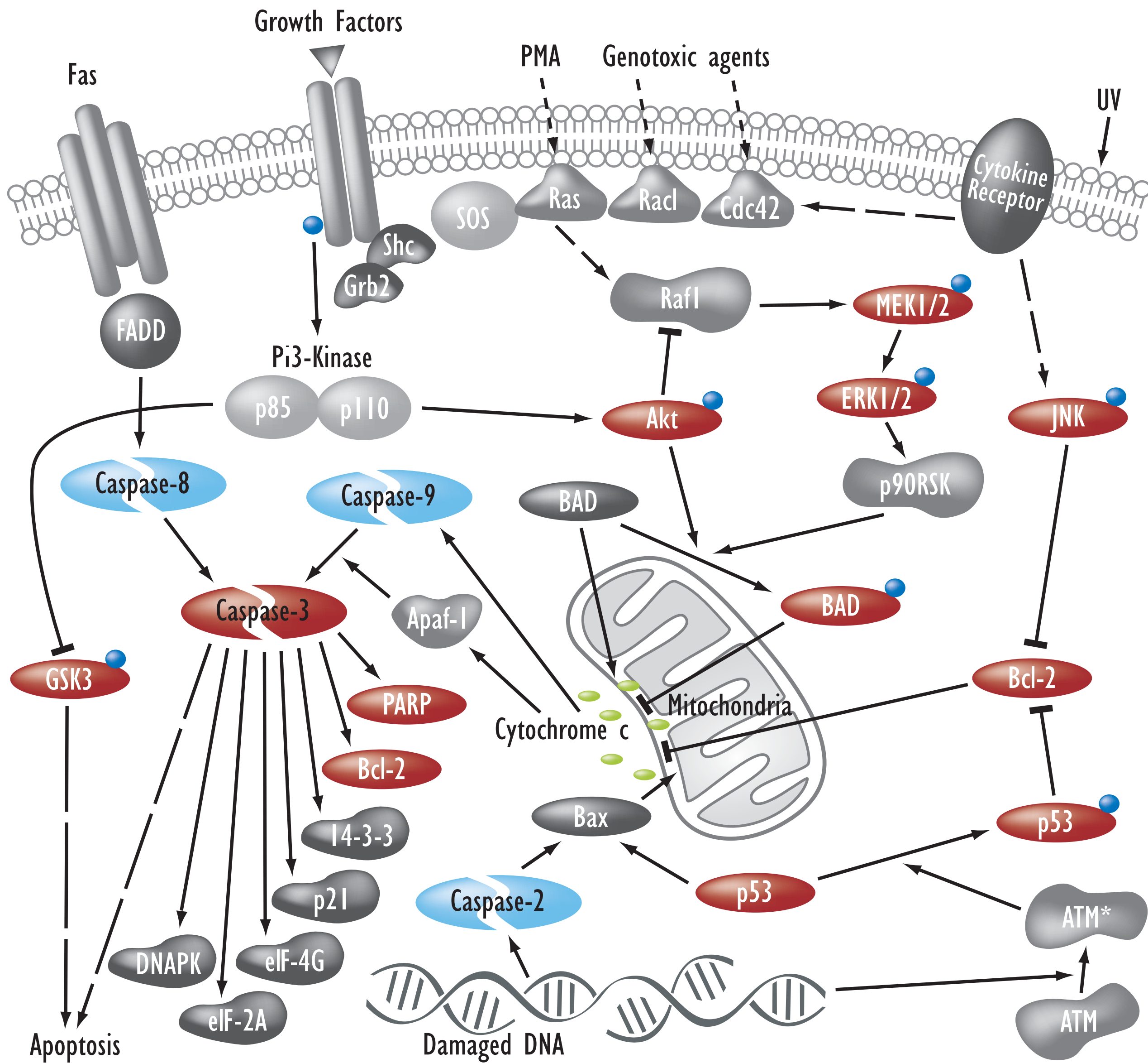
	Lysates (µg)	Positive		Negative		P/N
		Average	%CV	Average	%CV	
pp38	0			145	16	
	0.31	711	2	243	3	2.9
	0.63	1,070	0	324	12	3.3
	1.25	2,147	1	327	1	6.6
	2.5	4,513	7	255	7	17.7
	5	10,763	8	225	3	47.8
	10	35,186	9	181	0	194.4
	20	72,986	1	183	1	398.8
pJNK	0			303	4	
	0.31	1,608	4	412	1	3.9
	0.63	2,427	7	507	4	4.8
	1.25	4,303	2	554	0	7.8
	2.5	7,582	7	575	2	13.2
	5	10,187	14	640	2	15.9
	10	15,167	1	731	0	20.8
pERK1/2	0			193	4	
	0.02	1,003	3	198	9	5.1
	0.04	1,731	3	206	1	8.4
	0.08	2,569	5	213	5	12.1
	0.16	4,857	2	236	1	20.6
	0.31	7,694	2	283	1	27.2
	0.63	9,391	3	356	4	26.4
	1.25	8,828	4	347	1	25.4
	2.5	10,067	6	297	4	34.0
	5	10,505	6	237	11	44.3
	10	9,404	1	203	5	46.3





Logarithmically growing HEK293 cells were treated with UV + Calyculin A (positive) or rapamycin (negative). Whole cell lysates were added to MSD MULTI-SPOT 4-spot plates (pre-coated with capture antibody) and analyzed in a multiplexed sandwich assay.

Apoptosis Pathways



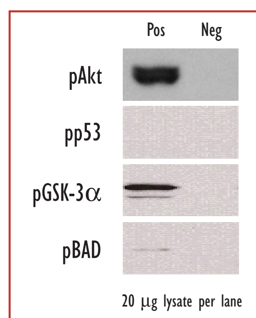
Phosphorylation modification | Targets analyzed in MSD multiplex formats | Cleavage modification of Caspase

Inhibitory modification | Multi-step pathway

Multiplexed Phospho-Akt, Phospho-p53, Phospho-GSK-3α and Phospho-BAD in Jurkat Whole Cell Lysates

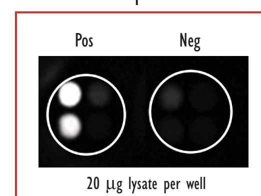
	Lysates (μg)	Positive		Negative		P/N
		Average	%CV	Average	%CV	
pAkt	0	274	21	259	4	
	10	25,950	0	1,637	2	15.9
	20	57,563	0	3,633	2	15.8
pp53	0	234	7	219	3	
	10	494	2	459	2	1.1
	20	493	5	433	4	1.1
pGSK-3α	0	269	0	275	2	
	10	10,620	4	690	1	15.4
	20	16,629	5	841	2	19.8
pBAD	0	222	13	210	4	
	10	1,752	1	508	3	3.5
	20	2,956	4	560	3	5.3

Traditional Western Blots

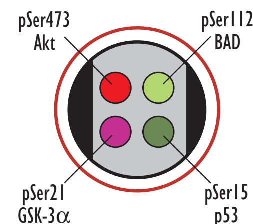


20 μg lysate per lane

MSD Experimental



20 μg lysate per well

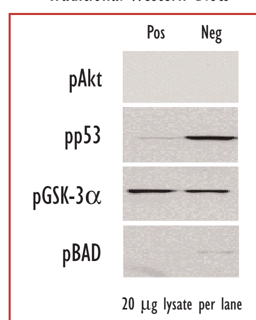


Logarithmically growing Jurkat cells (positive) were treated with LY294002 (50 μM) + staurosporine (1 μM) for 2.25 hr (negative). Whole cell lysates were added to MSD MULTI-SPOT 4-spot plates (pre-coated with capture antibody) and analyzed in a multiplexed sandwich assay.

in HT-29 Whole Cell Lysates

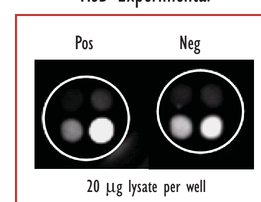
	Lysates (μg)	Positive		Negative		P/N
		Average	%CV	Average	%CV	
pAkt	0	275	14	287	2	
	10	1,458	4	1,981	4	0.7
	20	984	5	1,968	3	0.5
pp53	0	229	10	235	2	
	10	56,382	1	12,879	3	4.4
	20	83,826	5	13,721	3	6.1
pGSK-3α	0	293	5	294	1	
	10	6,309	3	8,476	5	0.7
	20	6,864	6	9,308	5	0.7
pBAD	0	243	6	225	5	
	10	2,899	2	2,354	1	1.2
	20	3,686	0	2,440	4	1.5

Traditional Western Blots



20 μg lysate per lane

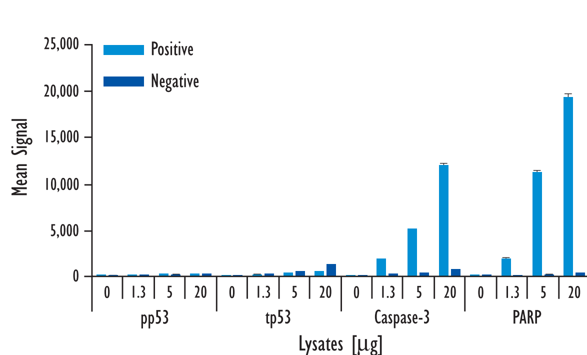
MSD Experimental

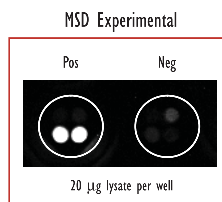


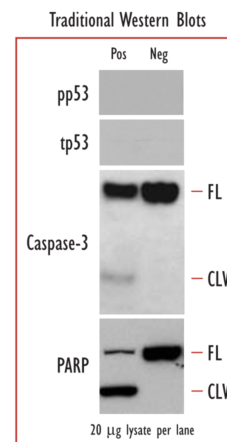
20 μg lysate per well

Growing HT-29 cells (negative) were harvested 1 hr after UV irradiation (40 mJ/cm^2) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4-spot plates (pre-coated with capture antibody) and analyzed in a multiplexed sandwich assay.

Multiplexed Phospho-p53, Total-p53, Cleaved Caspase-3 and Cleaved PARP in Jurkat Whole Cell Lysates

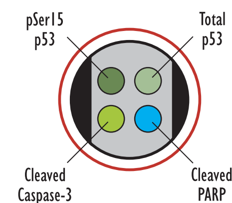




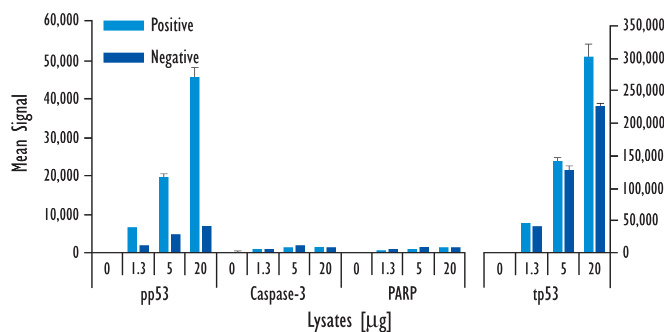


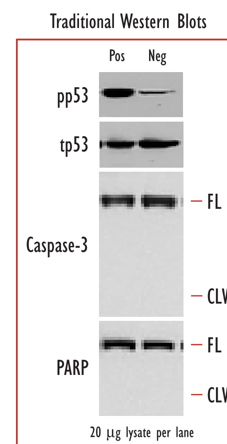
	Lysates (µg)	Positive		Negative		P/N
		Average	%CV	Average	%CV	
pp53	0	65	27	56	36	
	1.3	160	9	124	18	1.3
	5	256	7	185	17	1.4
	20	304	3	239	9	1.3
tp53	0	63	33	59	17	1.1
	1.3	179	13	216	9	0.8
	5	327	8	506	8	0.6
	20	479	1	1,313	0	0.4
Caspase-3	0	154	3	159	3	
	1.3	1,909	1	266	5	7.2
	5	5,113	1	424	4	12.1
	20	11,972	3	724	3	16.5
PARP	0	54	58	54	52	
	1.3	1,929	3	108	11	17.8
	5	11,244	2	177	9	63.5
	20	19,398	2	358	2	54.1

Logarithmically growing Jurkat cells (negative) were treated with etoposide (25 µM; 18 hr)(positive). Whole cell lysates were added to MSD MULTI-SPOT 4-spot plates (pre-coated with capture antibody) and analyzed in a multiplexed sandwich assay.



in HT-29 Whole Cell Lysates

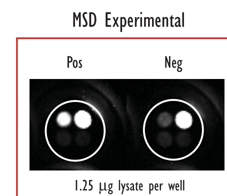




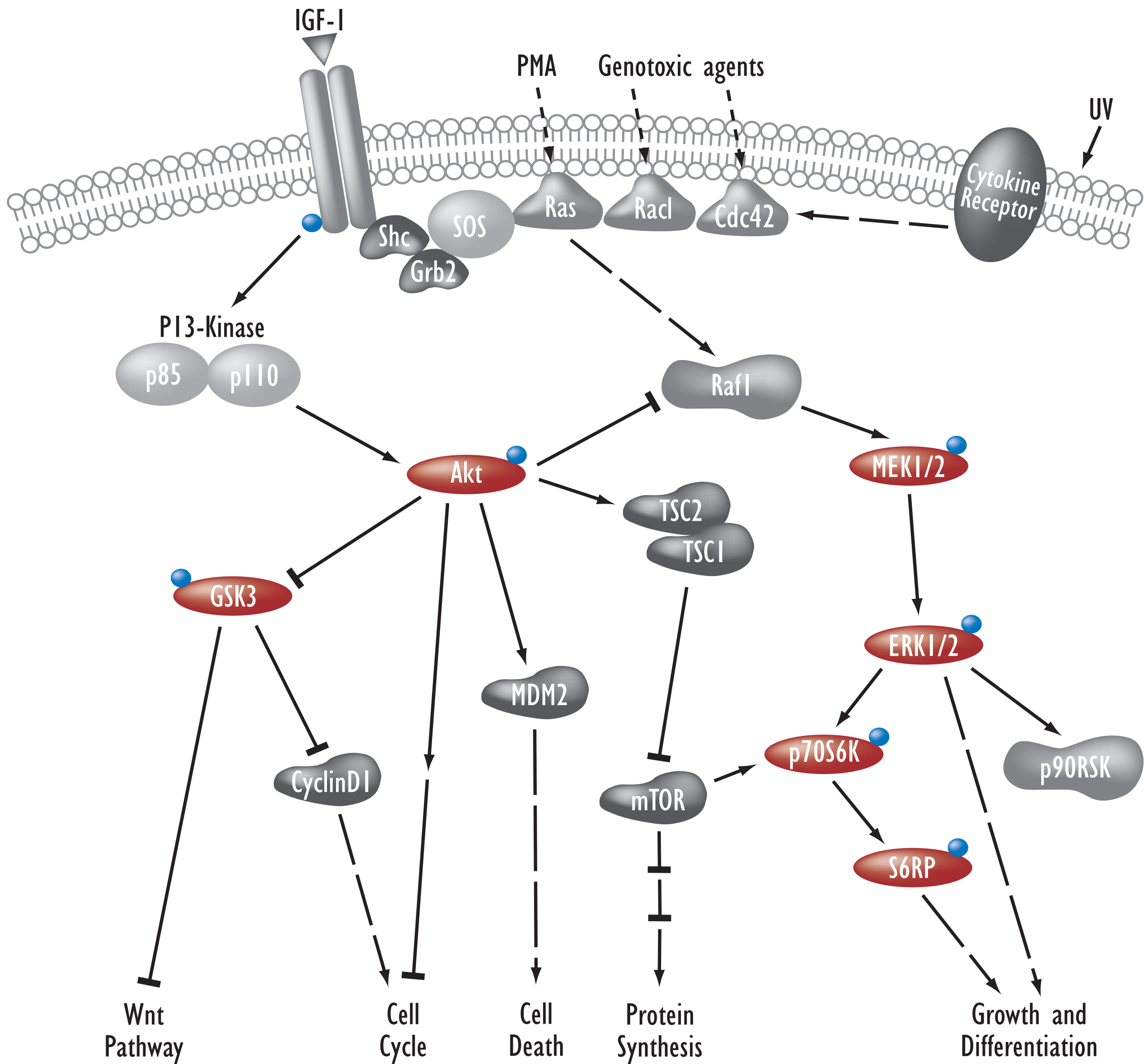
	Lysates (µg)	Positive		Negative		P/N
		Average	%CV	Average	%CV	
pp53	0	79	41	80	30	
	1.3	6,416	2	1,833	1	3.5
	5	19,743	4	4,693	4	4.2
	20	45,507	5	6,896	2	6.6
tp53	0	88	23	96	11	
	1.3	45,481	1	40,069	1	1.1
	5	141,558	3	126,178	7	1.1
	20	302,907	6	226,859	1	1.3
Caspase-3	0	189	10	192	14	
	1.3	725	4	931	4	0.8
	5	1,211	2	1,523	8	0.8
	20	1,128	4	1,254	2	0.9
PARP	0	87	15	91	24	
	1.3	588	6	776	7	0.8
	5	994	3	1,389	5	0.7
	20	1,084	5	1,172	9	0.9

Growing HT-29 cells (negative) were harvested 1 hr after UV irradiation (40 mJ/cm^2)(positive). Whole cell lysates were added to MSD MULTI-SPOT 4-spot plates (pre-coated with capture antibody) and analyzed in a multiplexed sandwich assay.

MSD Experimental



Akt / mTOR Signaling Pathway



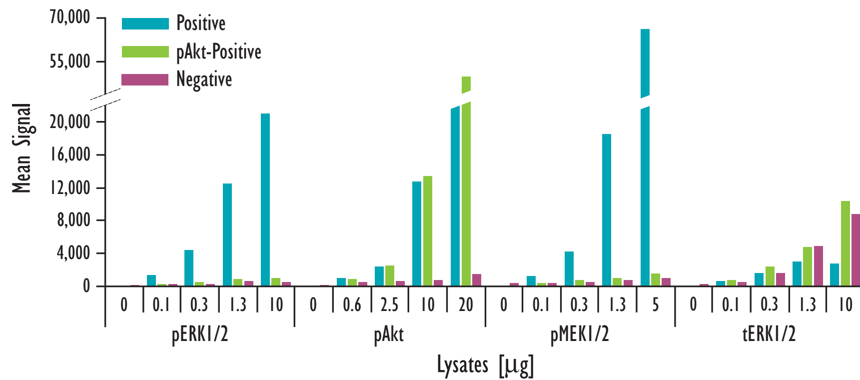
Phosphorylation modification

Targets analyzed in MSD multiplex formats

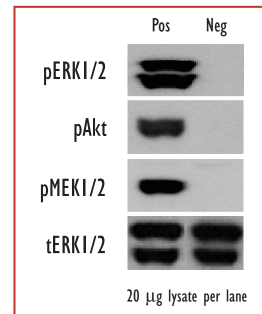
Inhibitory modification

Multi-step pathway

Multiplexed Phospho-ERK1/2, Phospho-Akt, Phospho-MEK1/2 and Total ERK1/2 Assay in Jurkat Whole Cell Lysates

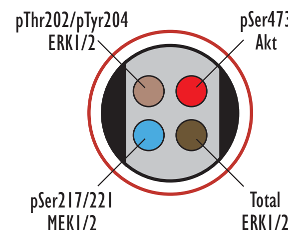


Traditional Western Blots

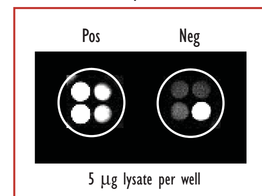


20 µg lysate per lane

	Lysates (µg)	Positive		pAkt-Positive		Negative		P2/N	P/N
		Average	%CV	Average	%CV	Average	%CV		
pERK1/2	0					172	2		
	0.08	1,274	1	266	3	186	5	1.4	6.9
	0.16	2,453	3	362	5	211	0	1.7	11.7
	0.3	4,343	3	493	5	286	10	1.7	15.2
	0.6	7,565	3	664	6	431	4	1.5	17.6
	1.3	12,430	2	779	2	595	4	1.3	20.9
	2.5	15,276	3	853	5	576	1	1.5	26.5
	5	18,484	2	912	5	551	0	1.7	33.5
pAkt	0					171	2		
	1.3	1,488	7	1,597	3	599	4	2.7	2.5
	2.5	2,385	2	2,486	11	544	0	4.6	4.4
	5	5,357	7	6,673	2	562	3	11.9	9.5
	10	12,663	6	13,337	14	703	3	19.0	18.0
	20	41,487	3	49,449	5	1,399	4	35.3	29.7
pMEK1/2	0					301	0		
	0.16	2,315	3	485	2	370	5	1.3	6.3
	0.3	4,119	2	703	31	421	2	1.7	9.8
	0.6	8,055	3	714	5	629	2	1.1	12.8
	1.3	18,424	4	905	3	751	4	1.2	24.5
	2.5	42,692	1	1,213	6	834	5	1.5	51.2
	5	66,286	1	1,543	2	925	1	1.7	71.7
	10	106,600	1	2,029	6	1,209	5	1.7	88.2
	20	132,204	1	2,455	30	1,258	3	2.0	105.1
tERK1/2	0					238	9		
	0.08	547	5	685	3	421	2	1.6	1.3
	0.16	904	4	1,333	4	818	3	1.6	1.1
	0.3	1,504	5	2,320	6	1,535	6	1.5	1.0
	0.6	2,161	3	3,578	6	2,683	4	1.3	0.8
	1.3	3,006	4	4,755	6	4,867	2	1.0	0.6
	2.5	2,381	4	6,269	10	6,202	10	1.0	0.4
	5	2,764	2	8,380	8	7,271	0	1.2	0.4



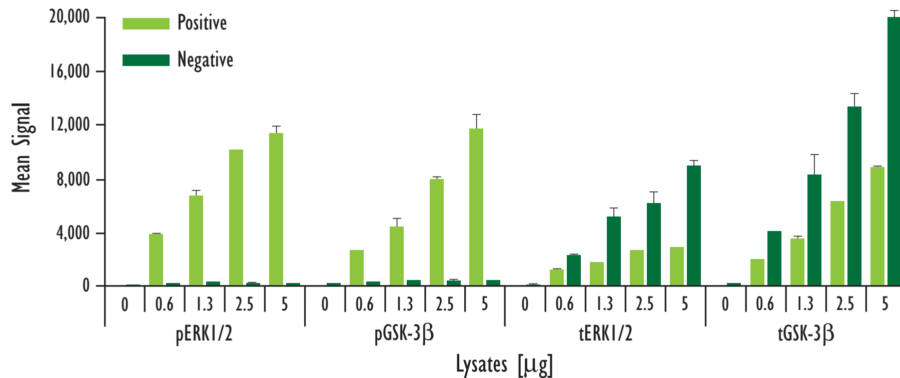
MSD Experimental



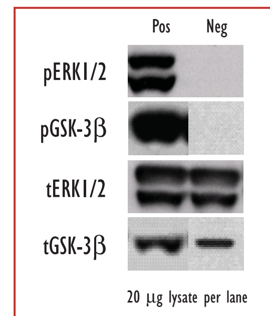
5 µg lysate per well

Logarithmically growing Jurkat cells (positive phospho-target pAkt only) were treated with PMA (200 nM; 15 min)(positive phospho-targets pERK1/2, pAkt, pMEK1/2) or LY294002(50 µM; 2.25 hr)(negative). Whole cell lysates were added to MSD MULTI-SPOT 4-spot plates (pre-coated with capture antibody) and analyzed in a multiplexed sandwich assay.

Multiplexed Phospho-ERK1/2, Phospho-GSK-3β, Total ERK1/2 and Total GSK-3β Assay in Jurkat Whole Cell Lysates

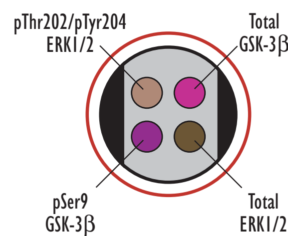


Traditional Western Blots

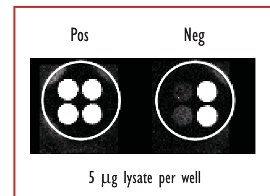


20 μg lysate per lane

	Lysates (μg)	Positive		Negative		P/N
		Average	%CV	Average	%CV	
pERK1/2	0			117	8	
	0.6	3,865	3	242	8	16.0
	1.3	6,760	6	277	3	24.4
	2.5	10,114	1	273	4	37.1
	5	11,415	4	274	0	41.7
pGSK-3β	0			197	10	
	0.6	2,623	1	351	1	7.5
	1.3	4,450	14	421	5	10.6
	2.5	7,990	2	469	7	17.0
	5	11,749	9	466	0	25.2
tERK1/2	0			153	11	
	0.6	1,226	4	2,357	4	0.5
	1.3	1,753	1	5,218	12	0.3
	2.5	2,613	2	6,212	13	0.4
	5	2,907	0	8,907	6	0.3
tGSK-3β	0			203	16	
	0.6	1,979	2	4,049	1	0.5
	1.3	3,523	6	8,322	19	0.4
	2.5	6,290	0	13,419	7	0.5
	5	8,872	1	19,990	3	0.4



MSD Experimental



5 μg lysate per well

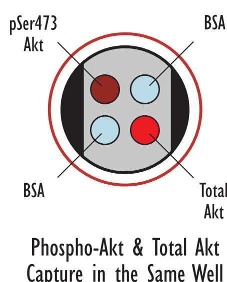
Logarithmically growing Jurkat cells were treated with PMA (200 nM; 15 min)(positive) or LY294002 (50 μM; 2.25 hr) (negative). Whole cell lysates were added to MSD MULTI-SPOT 4-spot plates (pre-coated with capture antibody) and analyzed in a multiplexed sandwich assay.

Quantification of the % Phosphoprotein in a Sample: Phosphorylated and Total Assay in the Same Well

- For demonstration purposes calculations for % phosphoprotein are shown here using data from the MSD MULTI-SPOT Phospho/Total Akt assay.
- The untreated cell lysates used here were derived from growing Jurkat cells and are expected to express phosphorylated Akt. Treating the cells with the LY294002 inhibitor is expected to inhibit phosphorylated Akt production but not affect the level of total Akt.

MSD MULTI-SPOT Phosphorylated and Total Akt Assay

Jurkat Lysates (µg)	pAkt Untreated			pAkt Treated			U-T	U/T	Total Akt Untreated			Total Akt Treated			U-T	U/T
	Average	StdDev	%CV	Average	StdDev	%CV			Average	StdDev	%CV	Average	StdDev	%CV		
0	89	16	18	112	16	14			220	44	20	243	44	18		
0.3	584	87	15	158	15	9	426	3.7	1,705	255	15	3,241	255	8	-1,537	0.5
0.6	983	170	17	169	21	12	815	5.8	3,286	89	3	5,853	89	2	-2,567	0.6
1.3	1,373	208	15	269	12	4	1,105	5.1	5,629	1,109	20	10,390	1,109	11	-4,762	0.5
2.5	3,124	742	24	294	28	9	2,830	10.6	11,701	1,050	9	19,325	1,050	5	-7,624	0.6
5	6,087	3,088	51	485	11	2	5,602	12.6	21,189	3,072	14	34,547	3,072	9	-13,359	0.6
10	15,169	3,718	25	732	60	8	14,438	20.7	33,731	4,094	12	52,575	4,094	8	-18,844	0.6
20	29,258	4,939	17	1,173	189	16	28,085	25.0	44,540	14	0	71,846	14	0	-27,307	0.6



Phospho-Akt & Total Akt Capture in the Same Well

Lysates (µg)	% pAkt Untreated	% pAkt Treated
0	57	63
0.3	51	9
0.6	46	6
1.3	39	5
2.5	42	3
5	45	3
10	62	3
20	79	3

Due to the difference in protein abundance in a particular sample, determination of the linear assay range for each protein is recommended. The linear range determined for Akt in Jurkat cell lysates is highlighted.

Calculation: (2X phospho signal*/phospho signal+ total signal) X 100**

* The numerator is 2X the phospho signal since the phosphorylated species is captured by both antibodies; only 1/2 on the phospho spot.

** The denominator is "phospho signal + total signal" since the actual "total" is all of the material detected on both spots.

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Conclusions

1. We present highly specific multiplexed assays for simultaneous detection of phosphorylated protein members of MAPK, apoptosis and Akt/mTOR pathways. The methods we present are general. Thus, multiple phosphoprotein and apoptosis members of signaling pathways can be assayed simultaneously in a single well in short (1-3 hr) incubation periods. The assays combine specific antibodies immobilized on MSD MULTI-SPOT plates combined with cocktails of detection antibodies labeled with electrochemiluminescent reporters.

2. Simultaneous quantification of the phosphorylated and total protein in the same well results in a simple, high-throughput method for estimating the percentage of the pool that is phosphorylated in a sample.

3. MULTI-ARRAY technology-based assays are powerful replacements for established methods because the assays are highly quantitative and save time and labor compared to existing techniques.