MAPK Cascade as a Signaling Convergence Point in Cancer Therapy: Simultaneous Detection of Activated p38, ERK and JNK

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MSD®

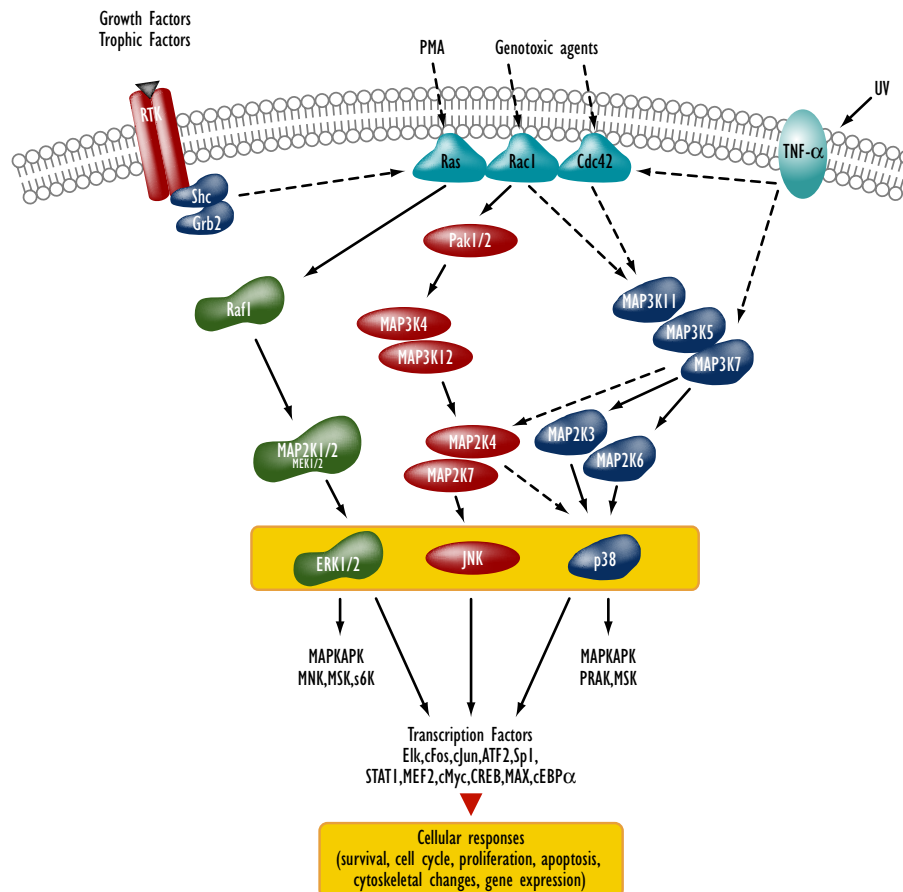Meso Scale Discovery
A division of Meso Scale Diagnostics, LLC.

MAPK Cascade as a Signaling Convergence Point in Cancer Therapy: Simultaneous Detection of Activated p38, ERK and JNK

❶ Abstract

The mitogen-activated protein kinase (MAPK) pathways, through major groupings like p38, ERK and JNK, constitute interrelated signal transduction cascades that regulate several diseases. In meeting the challenges of system biology and linear drug discovery, we present MSD's MULTI-SPOT® platform for simultaneous detection of phosphorylated forms of p38(T180/Y182), ERK1/2(T202/Y204)/(T185/Y187) and JNK(T183/Y185). The assays were performed in multiplex format and showed high sensitivity; phospho-p38 was quantifiable at attomoles, or from about 5,000 stimulated cells. High signal/background ratios were registered for phospho-p38 and phospho-JNK targets in UV treated HEK293 or NIH3T3 cells, and from the phospho-ERK1/2 target in Phorbol-ester treated Jurkat cells. Total pools of ERK1/2 and p38 were also detected quantitatively from cell lysates in the same well. These tools enable the rapid identification of drug candidates and promote the comprehensive understanding of the role of MAPK signaling pathway proteins.
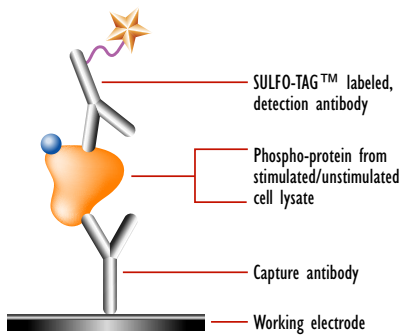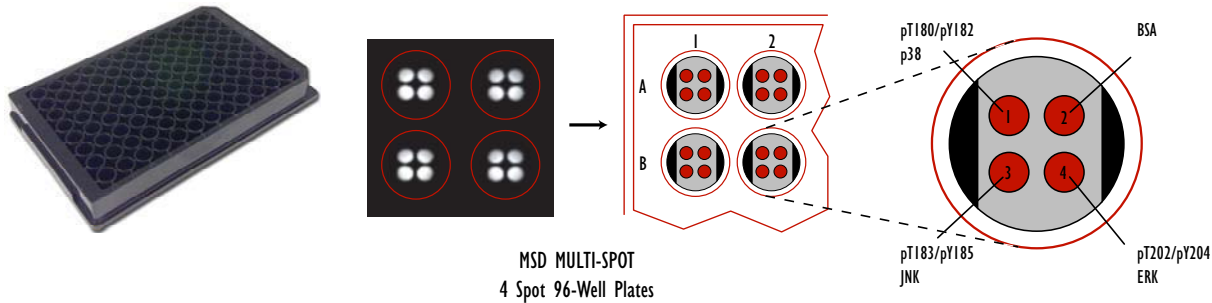
❷ MAPK Pathway Convergence: ERK1/2, JNK and p38

❸ MSD MULTI-ARRAY™ and MULTI-SPOT® Plates

MSD MULTI-SPOT
4 Spot 96-Well Plates

pT180/pY182 p38

BSA

pT183/pY185 JNK

pT202/pY204 ERK

SULFO-TAG™ labeled, detection antibody

Phospho-protein from stimulated/unstimulated cell lysate

Capture antibody

Working electrode

1. MULTI-SPOT 4 Spot 96-Well Plates precoated with capture antibodies are blocked with 200μL of MSD Blocker-A for 1h and washed with TBS.
2. 25μL of cell lysates are added to the wells and incubated for 3h at RT or overnight at 4°C with shaking, followed by washing with TBS.
3. 25μL MSD SULFO-TAG labeled antibodies (in Antibody dilution buffer) are added to the wells and incubated for 1h with shaking, followed by washing with TBS.
4. 150μL MSD Read Buffer T (with surfactant) are added to the wells and analyzed on the SECTOR™ 6000 instrument.
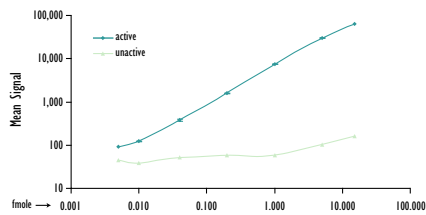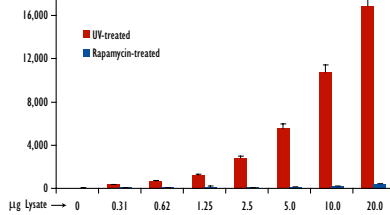
❹ MSD Singleplex Phospho-p38 (T180/Y182) assay

in purified recombinant preps

| Pure fmole	Active		Unactive		Active/ Unactive
	Ave.	%CV	Ave.	%CV	
0.0			48	22	
0.005	92	2	45	9	2.0
0.01	123	14	39	24	3.2
0.04	379	5	53	9	7.2
0.2	1,575	2	58	7	27.1
1	7,416	3	60	44	124.6
5	30,269		104	16	292.5
15	62,617	0	164	13	383.0

in HEK293 Whole Cell Lysates

HEK293 μg	UV-Treated		RP-Treated		Active/ Unactive
	Ave.	%CV	Ave.	%CV	
0.0			36	8	
0.31	334	7	45	27	7.5
0.62	623	15	47	24	13.3
1.25	1,271	1	123	69	10.3
2.5	2,852	5	85	7	33.6
5.0	5,531	8	124	13	44.8
10.0	10,759	6	199	5	54.1
20.0	16,929	5	402	5	42.1

Traditional Western: 20 μg protein per lane

in NIH3T3 Whole Cell Lysates
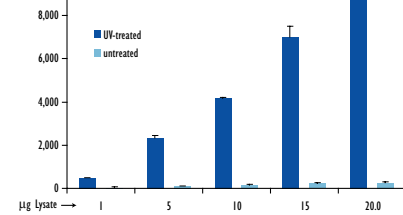
NIH3T3 μg	UV-Treated		Untreated		Active/ Unactive
	Ave.	%CV	Ave.	%CV	
1.0	488	3	53	17	9.3
5.0	2,329	5	114	8	20.4
10.0	4,159	2	155	15	26.9
15.0	7,007	7	235	13	29.8
20.0	9,467	0	279	4	34.0

Traditional Western: 20 μg protein per lane
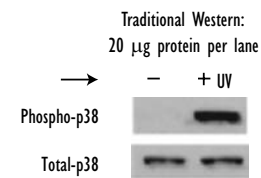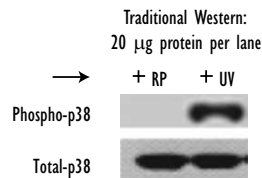
Growing HEK293 cells were treated with Rapamycin (1 μM; 3 hr)(+RP), while serum deprived HEK293 cells were harvested 30 minutes after UV irradiation (40mJ/cm^2)(+UV). Serum deprived NIH3T3 cells (-) were harvested 30 minutes after UV irradiation (40mJ/cm^2)(+UV). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with phospho-p38 antibody on one of the four spatially distinct electrodes per well. Phosphorylated p38 was detected with 10 nM anti-p38 antibody labeled with MSD SULFO-TAG reagents.
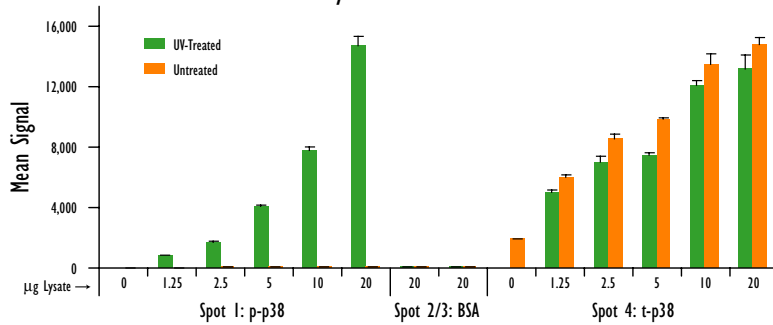
❺ MSD Multiplexed Phospho-p38 / Total-p38 assay

in NIH3T3 Whole Cell Lysates

Legend: UV-Treated; Untreated

Mean Signal vs µg Lysate → 0, 1.25, 2.5, 5, 10, 20, 20, 20, 0, 1.25, 2.5, 5, 10, 20

Spot 1: p-p38   Spot 2/3: BSA   Spot 4: t-p38

Spot	NIH3T3 µg	UV-treated Ave.	%CV	Untreated Ave.	%CV	Active/ Unactive
1:pp38	0.0			27	27	
1:pp38	1.25	847	2	34	13	25.2
1:pp38	2.5	1,706	5	47	35	36.0
1:pp38	5.0	4,071	2	66	10	62.0
1:pp38	10.0	7,821	3	81	10	97.0
1:pp38	20.0	14,750	5	99	6	148.5
2:BSA	20.0	52	35	47	30	
3:BSA	20.0	63	23	55	8	
4:tp38	0.0			1,916	2	
4:tp38	1.25	5,051	3	6,042	2	0.8
4:tp38	2.5	7,050	5	8,603	3	0.8
4:tp38	5.0	7,505	2	9,877	1	0.8
4:tp38	10.0	12,165	2	13,528	5	0.9
4:tp38	20.0	13,213	7	14,811	3	0.9

MULTI-ARRAY Technology

− UV   + UV

● High signal
○ Background

Predicted / Experimental

in HEK293 Whole Cell Lysates

Legend: UV-Treated; Rapamycin-treated

Mean Signal vs µg Lysate → 0, 1.25, 2.5, 5, 10, 20, 20, 20, 0, 1.25, 2.5, 5, 10, 20

Spot 1: p-p38   Spot 2/3: BSA   Spot 4: t-p38

Spot	HEK293 µg	UV-treated Ave.	%CV	RP-treated Ave.	%CV	Active/ Unactive
1:pp38	0.0			82	19	
1:pp38	1.25	604	6	113	4	5.3
1:pp38	2.5	1,243	4	126	6	9.9
1:pp38	5.0	3,028	6	142	12	21.4
1:pp38	10.0	5,578	6	192	12	29.1
1:pp38	20.0	10,011	11	319	0	31.4
2:BSA	20.0	159	16	121	5	
3:BSA	20.0	164	4	135	9	
4:tp38	0.0			1,721	12	
4:tp38	1.25	4,199	14	4,013	2	1.0
4:tp38	2.5	5,695	7	5,376	1	1.1
4:tp38	5.0	7,505	9	6,609	1	1.1
4:tp38	10.0	8,499	4	8,101	6	1.0
4:tp38	20.0	9,584	2	9,239	1	1.0
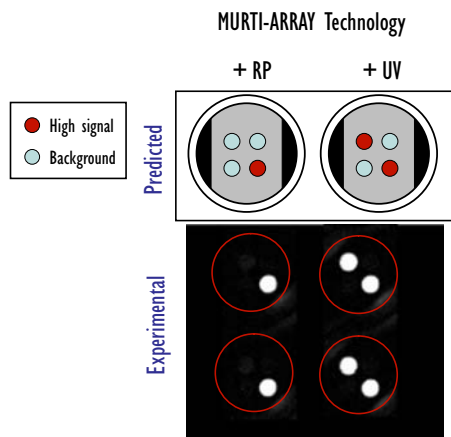
MURTI-ARRAY Technology

+ RP   + UV

● High signal
○ Background

Predicted / Experimental

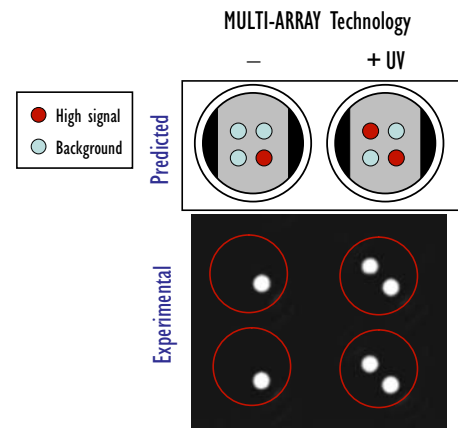Meso Scale Discovery
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MAPK Cascade as a Signaling Convergence Point in Cancer Therapy: Simultaneous Detection of Activated p38, ERK and JNK
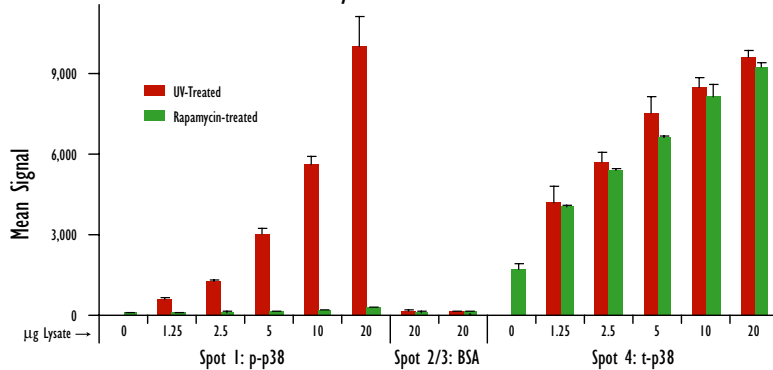
❻ MSD Singleplex Phospho-ERK1/2 (T/Y:202/204;185/187) assay
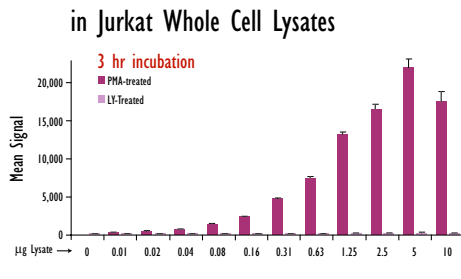
in Jurkat Whole Cell Lysates — 3 hr incubation

Traditional Western: 20 μg protein per lane

in Jurkat Whole Cell Lysates — overnight incubation

Jurkat μg	PMA-Treated Ave	%CV	LY-Treated Ave	%CV	S/B	Active/Unactive
0			87	11		
0.01	280	3	89	12	3.2	3.1
0.02	449	5	81	6	5.2	5.6
0.039	716	5	68	11	8.3	10.6
0.078	1,398	1	85	14	16.1	16.4
0.16	2,404	1	92	7	27.7	26.2
0.31	4,652	3	120	3	53.7	38.9
0.63	7,376	4	141	1	85.1	52.2
1.25	13,072	2	181	2	150.8	72.1
2.5	16,483	4	219	1	190.2	75.4
5	21,853	5	257	7	252.1	84.9
10	17,330	8	244	4	200	71.1
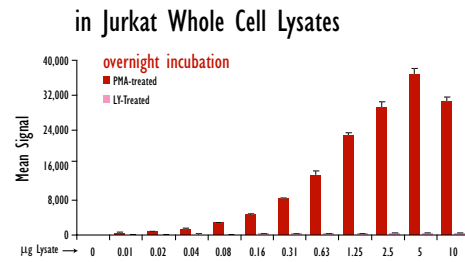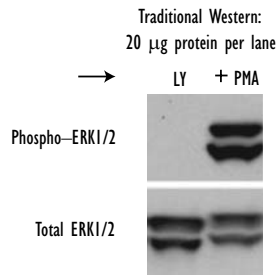
Jurkat μg	PMA-Treated Ave	%CV	LY-Treated Ave	%CV	S/B	Active/Unactive
0			68	27		
0.01	449	4	74	5	6.6	6
0.02	771	2	67	11	11.3	11.4
0.04	1,334	3	82	17	19.5	16.3
0.08	2,661	2	82	9	38.9	32.6
0.16	4,537	5	100	5	66.4	45.4
0.31	8,316	1	122	5	121.7	68.2
0.63	13,688	6	177	6	200.3	77.5
1.25	22,746	3	226	8	332.9	100.6
2.5	29,147	4	314	5	426.5	92.9
5	36,659	3	349	6	536.5	105.1
10	30,455	3	347	1	445.7	87.7

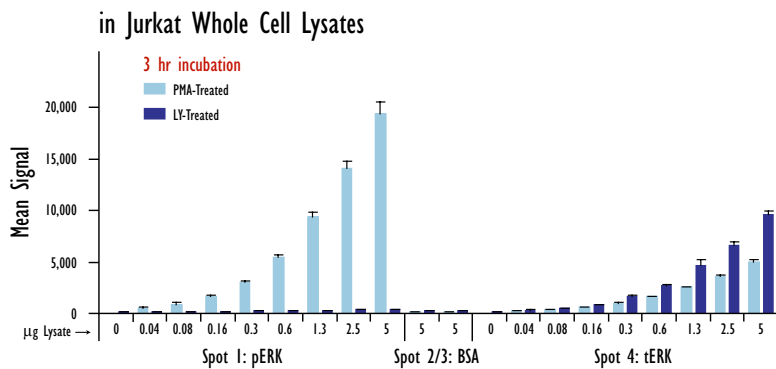Logarithmically growing Jurkat cells were treated with LY294002 inhibitor (50 μM; 2.25 hr)(LY) or PMA (200 nM; 15 minutes)(+PMA). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with anti ERK1/2 antibody on one of the four spatially distinct electrodes per well. Phosphorylated ERK1/2 was detected with 10 nM anti phospho-ERK1/2 antibody labeled with MSD SULFO-TAG reagents.
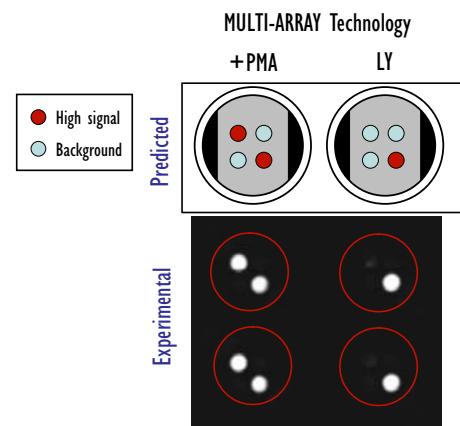
MAPK Cascade as a Signaling Convergence Point in Cancer Therapy: Simultaneous Detection of Activated p38, ERK and JNK

❼ MSD Multiplexed Phospho-ERK1/2 / Total-ERK1/2 assay

in Jurkat Whole Cell Lysates

3 hr incubation
- PMA-Treated
- LY-Treated

MULTI-ARRAY Technology

+PMA   LY

- High signal
- Background

Predicted

Experimental

Spot	Jurkat µg	PMA-treated Ave.	%CV	LY-Treated Ave.	%CV	Active/ Unactive
1:pERK	0			143	9	
1:pERK	0.02	343	6	154	10	2.2
1:pERK	0.04	511	4	134	5	3.8
1:pERK	0.08	849	22	145	5	5.9
1:pERK	0.16	1,660	3	148	5	11.2
1:pERK	0.31	2,979	6	181	4	16.4
1:pERK	0.63	5,447	4	217	5	25.1
1:pERK	1.25	9,324	5	267	7	34.9
1:pERK	2.5	14,091	5	317	1	44.5
1:pERK	5.0	19,386	6	385	4	50.4
2:BSA	5.0	154	7	215	5	
3:BSA	5.0	161	4	237	3	
4:tERK	0			151	9	
4:tERK	0.02	197	6	205	5	1.0
4:tERK	0.04	238	3	281	4	0.8
4:tERK	0.08	351	6	444	1	0.8
4:tERK	0.16	581	5	812	4	0.7
4:tERK	0.31	973	2	1,629	3	0.6
4:tERK	0.63	1,625	1	2,661	2	0.6
4:tERK	1.25	2,500	4	4,590	13	0.5
4:tERK	2.5	3,580	4	6,588	5	0.5
4:tERK	5.0	4,999	3	9,544	4	0.5

Logarithmically growing Jurkat cells were treated with LY294002 (50 nM; 2.25 hr)(-) or PMA (200 nM; 15 minutes)(+). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with anti ERK1/2 antibody on two of the four spatially distinct electrodes per well. Phosphorylated and total ERK1/2 were detected with 10 nM anti-total-ERK1/2 antibody labeled with MSD SULFO-TAG reagent.

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❽ MSD Singleplex Phospho-JNK (T183/Y185) assay

in HEK293 Whole Cell Lysates
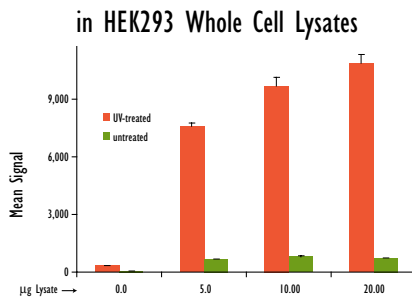
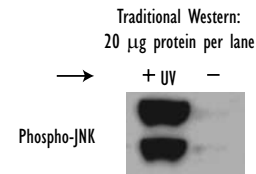| HEK293	UV-Treated		Untreated		Active/
μg	Ave.	%CV	Ave.	%CV	Unactive
0.0	358	2	41	14	
5.0	7,606	2	679	1	11.2
10.0	9,710	4	787	6	12.3
20.0	10,888	4	722	1	15.1

Traditional Western:
20 μg protein per lane

+ UV −

Phospho-JNK

Logarithmically growing HEK293 cells were treated with +/- UV. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with capture antibody on one of the four spatially distinct electrodes per well. Phosphorylated JNK was detected with 10 nM anti-phospho-JNK antibody labeled with MSD SULFO-TAG reagents.

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MAPK Cascade as a Signaling Convergence Point in Cancer Therapy: Simultaneous Detection of Activated p38, ERK and JNK

❾ MSD Multiplexed MAPK Panel : p-p38 / p-ERK / p-JNK

in HEK293 Whole Cell Lysates

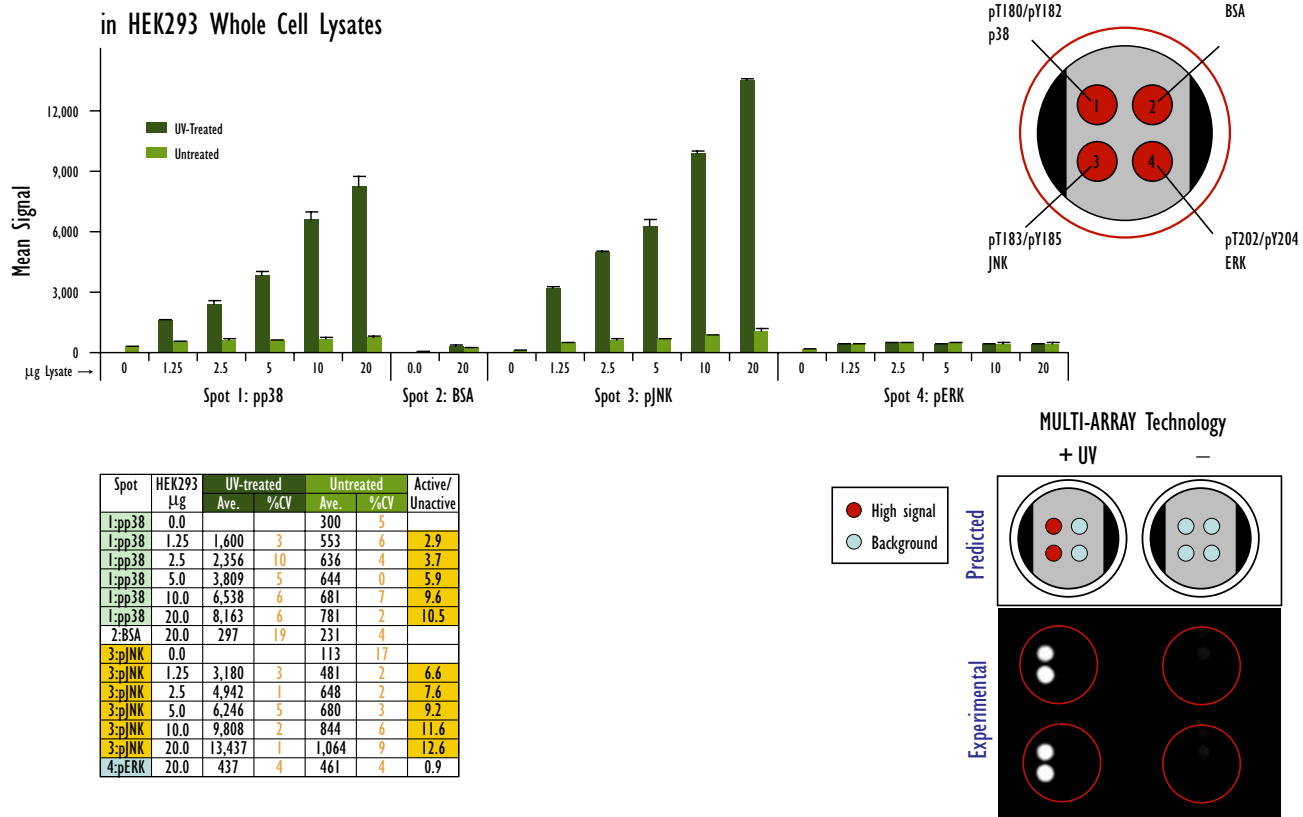| Spot	HEK293 μg	UV-treated Ave.	%CV	Untreated Ave.	%CV	Active/ Unactive
1:pp38	0.0			300	5	
1:pp38	1.25	1,600	3	553	6	2.9
1:pp38	2.5	2,356	10	636	4	3.7
1:pp38	5.0	3,809	5	644	0	5.9
1:pp38	10.0	6,538	6	681	7	9.6
1:pp38	20.0	8,163	6	781	2	10.5
2:BSA	20.0	297	19	231	4	
3:pJNK	0.0			113	17	
3:pJNK	1.25	3,180	3	481	2	6.6
3:pJNK	2.5	4,942	1	648	2	7.6
3:pJNK	5.0	6,246	5	680	3	9.2
3:pJNK	10.0	9,808	2	844	6	11.6
3:pJNK	20.0	13,437	1	1,064	9	12.6
4:pERK	20.0	437	4	461	4	0.9

MULTI-ARRAY Technology

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

❾ MSD Multiplexed MAPK Panel : p-p38 / p-ERK / p-JNK (continued)

in Jurkat Whole Cell Lysates
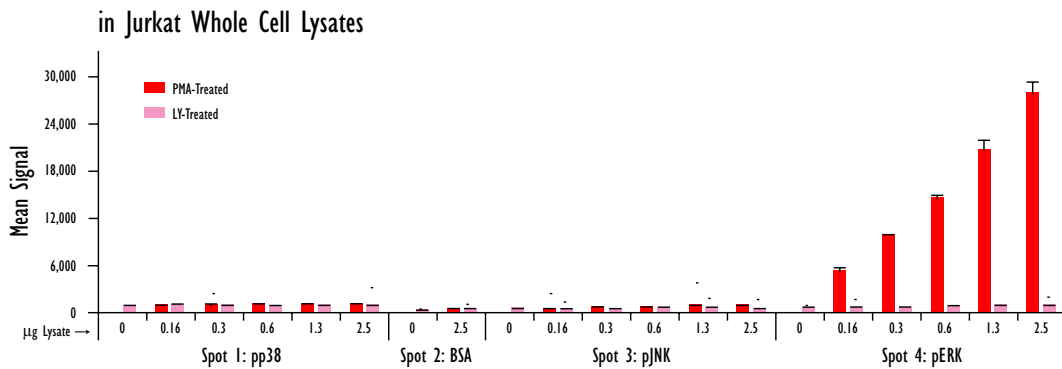
Spot	Jurkat μg	PMA-treated Ave.	PMA-treated %CV	LY-Treated Ave.	LY-Treated %CV	Active/ Unactive
1:pp38	0			808	9	
1:pp38	2.5	907	5	733	1	1.2
2:BSA	2.5	445	4	337	1	1.3
3:pJNK	0			354	11	
3:pJNK	2.5	801	3	457	6	1.8
4:pERK	0			486	17	
4:pERK	0.156	5,220	8	526	12	9.9
4:pERK	0.313	9,734	1	605	5	16.1
4:pERK	0.625	14,366	3	673	12	21.3
4:pERK	1.25	20,685	5	774	10	26.7
4:pERK	2.5	27,770	5	766	15	36.3
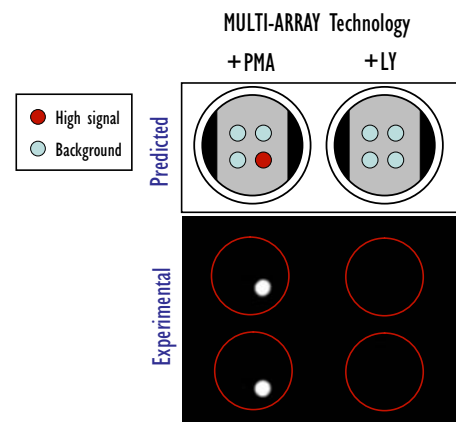
MULTI-ARRAY Technology

Logarithmically growing Jurkat cells were treated with LY294002 inhibitor (50 μM; 2.25 hr)(LY) or PMA (200 nM; 15 minutes)(+PMA). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with capture antibody on three of the four spatially distinct electrodes per well. Phosphorylated proteins were detected with MSD SULFO-TAG detection antibodies.

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❿ Conclusion

1. We present highly specific multiplexed assays for simultaneous detection of phosphorylated MAPK proteins, phospho-p38 (pT180/pY182), phospho-ERK1/2 (pT202/pY204 and pT185/pY187) and phospho-JNK (pT183/pY185).

2. These assays specifically identify phosphoproteins from human and murine cell lysates as well as recombinant purified proteins. The phospho-p38 detection assay identifies attomole levels of recombinant purified active protein with high sensitivity; signal / background ratio of about 380 (active vs inactive protein).

3. The phosphoprotein assays could be run in duplex format, thus simultaneously determining the phosphorylated and total levels of the target protein.

4. The methods we present are general, demonstrating that multiple phosphoprotein members of signaling pathways can be assayed simultaneously in a single well in short (3 hr) or long (overnight) incubation periods, using specific antibodies immobilized on MSD MULTI-SPOT plates.

5. The MULTI-ARRAY technology-based assays are useful supplements to gold-standard methods like western blots because these versatile assays are highly quantitative and easily automated, suitable for HTS, and save time and labor compared to existing techniques.

M S D ®

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