Multiplex Analysis of Akt/mTOR and JAK/STAT Signaling Pathways

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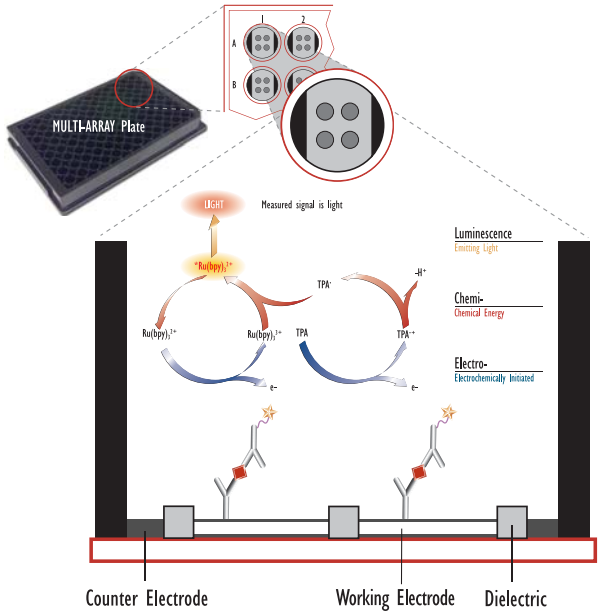
Phosphoproteins play a major role in cellular signaling throughout development and in adult organisms. The importance of these proteins in normal and disease states makes them ideal candidates for drug development both as therapeutic targets and as biomarkers of cellular behavior. The experimental characterization of phosphoproteins is made more efficient through the use of multiplex assays in microtiter plate formats. Here we present a number of multiplex, microtiter plate-based assays that facilitate the study of the Akt/mTOR pathway and the JAK/STAT pathway. The assays are very sensitive, affording detection from sub-microgram quantities of total protein and thus consistent with 384-well tissue culture applications. The results obtained with these multiplex assays are in agreement with conventional analysis by western blot with phospho-specific antibodies. Assay time is rapid and thus consistent with medium to high throughput workflows. The assays have been shown to work with a wide variety of cell types. Results from HeLa, Jurkat, HEK 293, MCF-7 and human T cells are shown here.

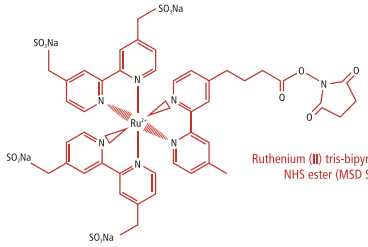
The MSD® Platform

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.

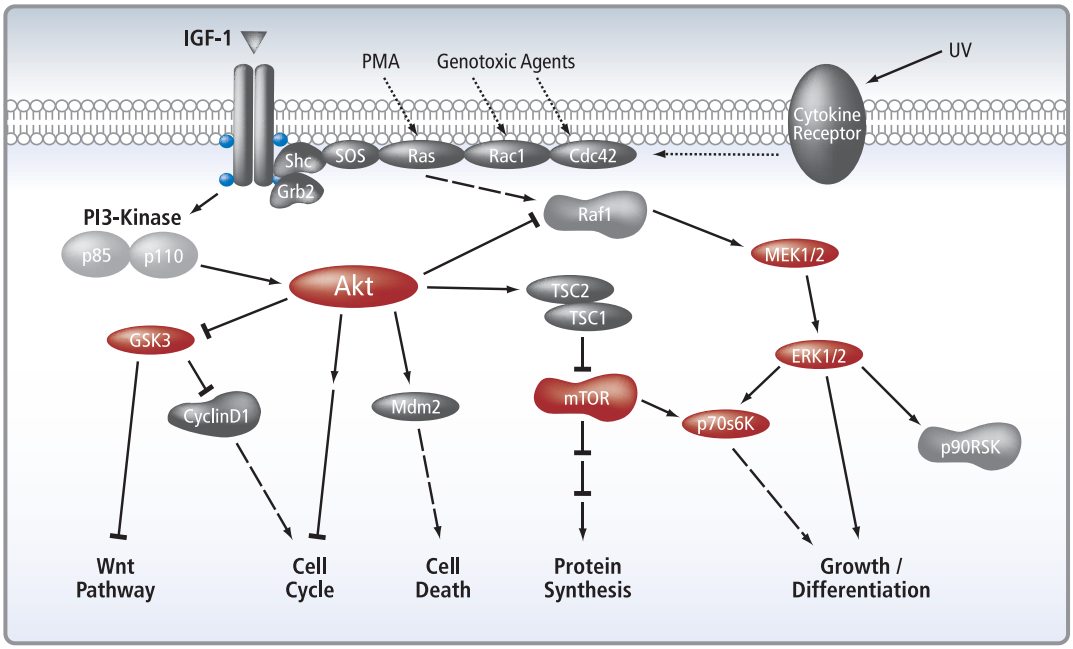


Electrochemiluminescence Features:

- Minimal background signals and high signal to background ratios - the stimulation mechanism (electricity) is decoupled from the signal (light)
- Proximity - only labels bound near the electrode surface are detected, enabling non-washed assays
- Flexibility - labels are stable, non-radioactive, and are conveniently conjugated to biological molecules
- Emission at ~620 nm - eliminating problems with color quenching
- Signal amplification - multiple excitation cycles of each label enhance light levels and improve sensitivity

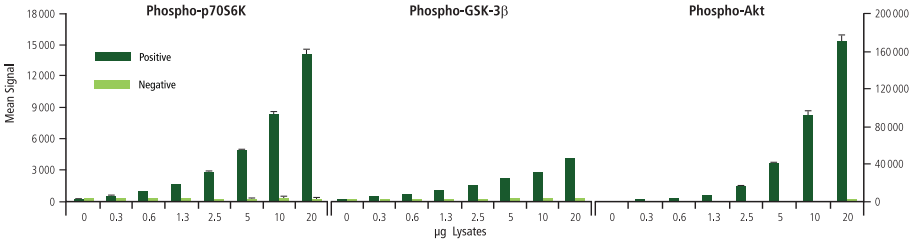


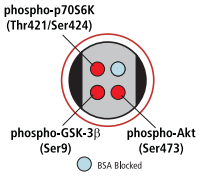
AKT/mTOR Signaling Pathway

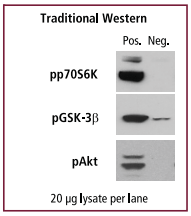


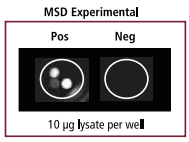
Available AKT/mTOR pathway phosphoprotein targets

Akt Signaling in Jurkat Cells: Simultaneous Analysis of Phospho-Akt (Ser473), Phospho-p70S6K (Thr421/Ser424), and Phospho-GSK-3β (Ser9)





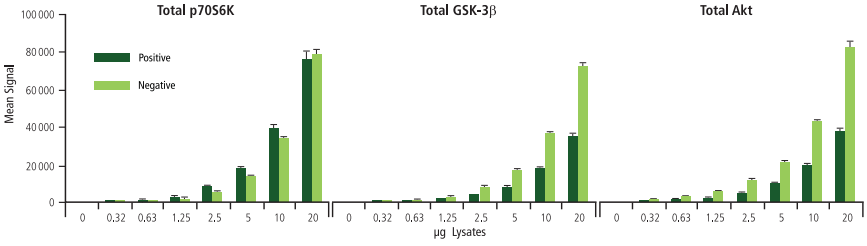


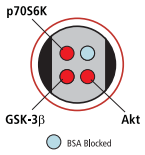


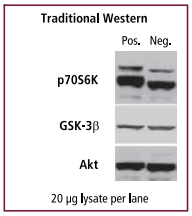
Logarithmically growing Jurkat cells were treated with LY294002 (50 µM) and staurosporine (1 µM; 2.5 hours) (negative) or PMA (200 nM, 15 minutes) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with anti-total p70S6K, anti-phospho-GSK-3β and anti-total Akt antibodies on three of the four spatially distinct electrodes per well. Phosphorylated p70S6K, GSK-3β and Akt were detected with anti-phospho-p70S6K, anti-total GSK-3β, and anti-phospho-Akt antibodies labeled with MSD SULFO-TAG reagent.

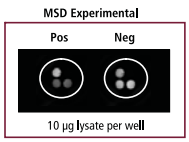
	Lysate (µg)	Positive Ave	Positive StdDev	Positive %CV	Negative Ave	Negative StdDev	Negative %CV	P/N
Phospho-p70S6K	0	163	9	5	186	16	9	
	0.3	475	32	7	175	7	4	2.7
	0.6	847	23	3	186	23	12	4.6
	1.3	1515	30	2	197	14	7	7.7
	2.5	2721	189	7	180	12	7	15.1
	5	4833	174	4	197	4	2	24.5
	10	8270	303	4	211	8	4	39.2
	20	14016	594	4	220	9	4	63.8
Phospho-GSK-3β	0	90	6	7	118	34	29	
	0.3	448	9	2	109	9	8	4.1
	0.6	664	11	2	146	18	13	4.5
	1.3	1011	35	3	144	12	8	7.0
	2.5	1539	87	6	133	10	7	11.6
	5	2126	129	6	166	6	3	12.8
	10	2778	263	9	166	9	5	16.8
	20	4082	86	2	176	5	3	23.2
Phospho-Akt	0	84	6	7	106	5	4	
	0.3	1168	55	5	132	19	15	8.8
	0.6	2542	165	6	151	13	9	16.8
	1.3	5944	163	3	154	1	0	38.5
	2.5	15660	1035	7	189	9	5	83.0
	5	39960	1310	3	270	16	6	147.8
	10	92247	4745	5	416	14	3	221.7
	20	170364	6518	4	758	49	6	224.8

Total Pools of Phosphoprotein are Constant in Jurkat Cells: Akt, p70S6K, and GSK-3β





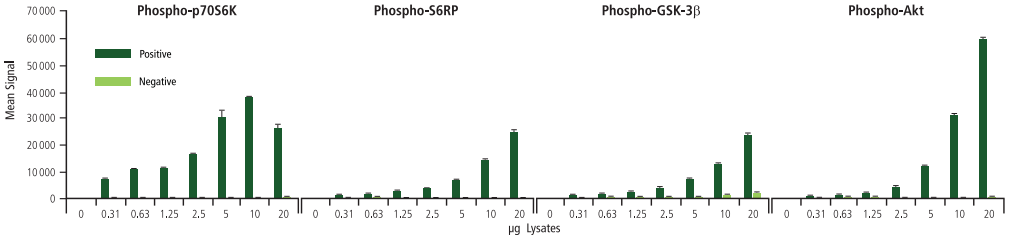


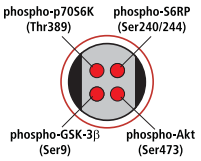


Jurkat cells were treated with LY294002 (50 µM, 2.5 hours) (negative) or with PMA (200 nM, 15 minutes) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with anti-total p70S6K, anti-total Akt, and anti-total GSK-3β antibodies on three of the four spatially distinct electrodes per well. p70S6K, Akt, and GSK-3β were detected with anti-total p70S6K, anti-total Akt, and anti-total GSK-3β antibodies labeled with MSD SULFO-TAG reagent.

	Lysate (µg)	Positive Ave	Positive StdDev	Positive %CV	Negative Ave	Negative StdDev	Negative %CV	P/N
Total p70S6K	0	417	14	3	419	18	4	
	0.32	999	14	1	725	66	9	1.4
	0.63	1697	51	3	1041	42	4	1.6
	1.25	3624	81	2	2198	103	5	1.6
	2.5	8326	274	3	5562	270	5	1.5
	5	17954	822	5	13848	483	3	1.3
	10	38980	1702	4	34141	567	2	1.1
	20	76314	4048	5	78165	3055	4	1.0
Total GSK-3β	0	146	8	5	142	9	6	
	0.32	504	10	2	899	18	2	0.6
	0.63	936	17	2	1723	31	2	0.5
	1.25	1832	119	6	3684	91	2	0.5
	2.5	4021	57	1	8315	200	2	0.5
	5	8396	278	3	17216	1033	6	0.5
	10	17919	691	4	36485	1202	3	0.5
	20	35041	1152	3	71893	2627	4	0.5
Total Akt	0	158	5	3	129	21	16	
	0.32	876	56	6	1784	75	4	0.5
	0.63	1596	73	5	3315	179	5	0.5
	1.25	2859	29	1	6245	400	6	0.5
	2.5	5285	311	6	12009	199	2	0.4
	5	10161	113	1	21572	459	2	0.5
	10	19457	811	4	42452	1324	3	0.5
	20	37367	1596	4	82518	3243	4	0.5

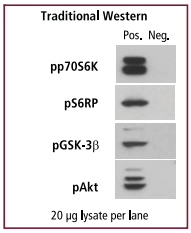
Akt and Downstream Targets Detected in the Same Well : Phospho-Akt (Ser473), Phospho-p70S6K (Thr389), Phospho-GSK-3β (Ser9) and Phospho-S6RP (Ser240/244)



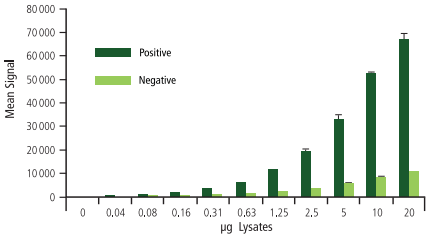


Growing MCF-7 cells were treated with LY294002 (50 µM, 2.5 hours) (negative) or IGF-1 (100 nM, 20 minutes) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with anti-total p70S6K, anti-phospho-S6RP, anti-phospho-GSK-3β, and anti-total Akt antibodies on each of the four spatially distinct electrodes per well. Phosphorylated p70S6K, S6RP, GSK-3β, and Akt were detected with anti-phospho-p70S6K, anti-total S6RP, anti-total GSK-3β, and anti-phospho-Akt antibodies labeled with MSD SULFO-TAG reagent.

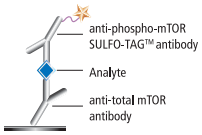
	Lysate (µg)	Positive			Negative			P/N
		Ave	StdDev	%CV	Ave	StdDev	%CV	
Phospho-p70S6K	0				84	4	4	
	0.31	7530	25	0	448	42	9	16.8
	0.63	10948	207	2	581	11	2	18.9
	1.25	11409	150	1	564	4	1	20.2
	2.5	16437	393	2	441	77	17	37.3
	5	30257	2763	9	360	36	10	84.2
	10	38034	362	1	490	11	2	77.6
	20	26092	1619	6	747	69	9	35.0
Phospho-S6RP	0				95	23	25	
	0.31	1388	24	2	497	26	5	2.8
	0.63	2013	13	1	636	35	5	3.2
	1.25	3027	44	1	570	33	6	5.3
	2.5	3989	213	5	381	28	7	10.5
	5	7174	264	4	344	21	6	20.9
	10	14510	532	4	360	23	6	40.3
	20	25047	885	4	484	24	5	51.7
Phospho-GSK-3β	0				102	18	18	
	0.31	1403	76	5	566	23	4	2.5
	0.63	2062	46	2	757	33	4	2.7
	1.25	2858	71	2	850	30	3	3.4
	2.5	4229	37	1	882	16	2	4.8
	5	7309	237	3	905	6	1	8.1
	10	13123	233	2	1585	113	7	8.3
	20	23860	510	2	2452	11	0	9.7
Phospho-Akt	0				99	10	10	
	0.31	1146	42	4	522	19	4	2.2
	0.63	1591	11	1	627	1	0	2.5
	1.25	2453	151	6	631	41	6	3.9
	2.5	4551	82	2	408	6	1	11.2
	5	12105	498	4	379	4	1	31.9
	10	31427	169	1	429	18	4	73.3
	20	59373	1136	2	848	26	3	70.1

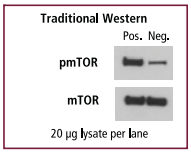


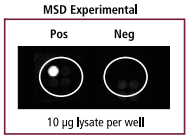
Ultrasensitive Detection of Phosphorylated mTOR (Ser2448) is Demonstrated in HEK293 Whole Cell Lysates



Lysate (µg)	Positive			Negative			P/N
	Ave	StdDev	%CV	Ave	StdDev	%CV	
0				115	0	0	
0.04	557	15	3	209	6	3	2.7
0.08	939	25	3	290	6	2	3.2
0.16	1705	46	3	406	1	0	4.2
0.31	3338	55	2	713	1	0	4.7
0.63	6142	62	1	1195	35	3	5.1
1.25	11512	170	1	1991	6	0	5.8
2.5	19249	982	5	3389	45	1	5.7
5	32943	1894	6	5726	433	8	5.8
10	52675	324	1	8474	355	4	6.2
20	66628	2674	4	10908	296	3	6.1

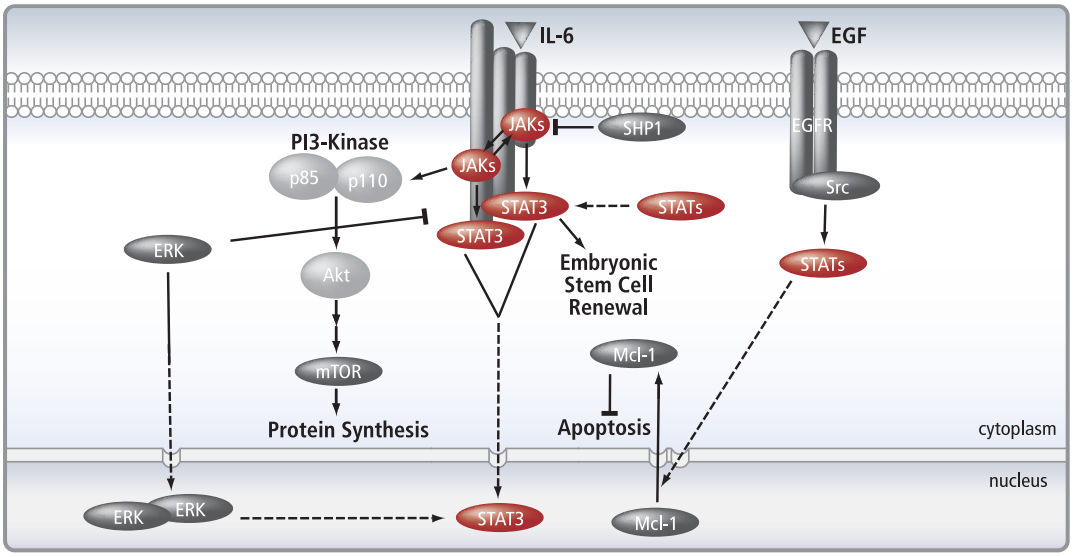






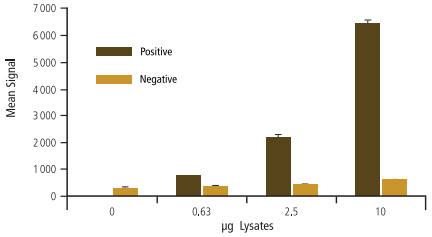
Growing HEK293 cells were treated with Wortmannin (100 nM, 3 hours) (negative) or PMA (1 µM, 30 minutes) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with anti-total mTOR antibody on one of the four spatially distinct electrodes per well. Phosphorylated mTOR was detected with anti-phospho-mTOR antibody labeled with MSD SULFO-TAG reagent.

JAK/STAT Signaling Pathway

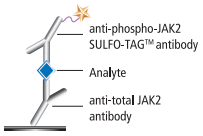


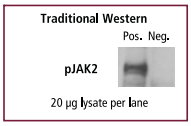
Available JAK/STAT pathway phosphoprotein targets

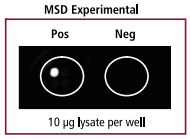
Phosphorylated JAK2 (Tyr1007/1008) is Quantified in T cells derived from Human PBMCs



Lysate (µg)	Positive			Negative			P/N
	Ave	StdDev	%CV	Ave	StdDev	%CV	
0				280	51	18	
0.63	754	10	1	364	26	7	2.1
2.5	2172	129	6	440	17	4	4.9
10	6476	117	2	613	13	2	10.6

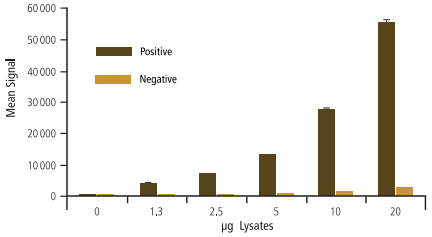




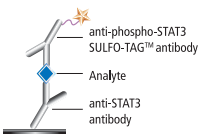


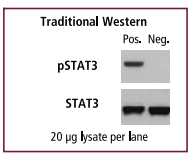
Starved human T cells (negative) were treated with IL-12 (10 ng/mL) and IFNα (1000 U/mL) for 30 minutes (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with anti-total JAK2 antibody on one of the four spatially distinct electrodes per well. Phosphorylated JAK2 was detected with anti-phospho-JAK2 antibody labeled with MSD SULFO-TAG reagent.

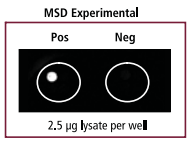
Phosphorylated STAT3 (Tyr705) Detected in HeLa Whole Cell Lysates



Lysate (µg)	Positive			Negative			P/N
	Ave	StdDev	%CV	Ave	StdDev	%CV	
0	201	10	5	205	8	4	
1.3	4118	84	2	563	14	3	7.3
2.5	6986	127	2	711	25	4	9.8
5	13320	35	0	1014	45	4	13.1
10	27389	699	3	1491	26	2	18.4
20	55332	858	2	2684	50	2	20.6

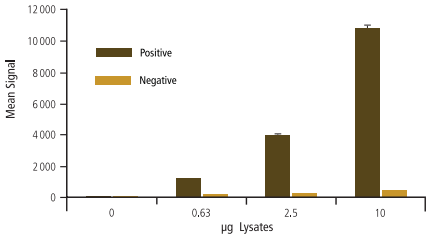




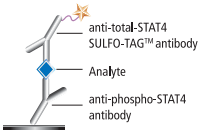


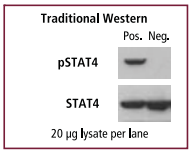
Confluent HeLa cells (negative) were pretreated with sodium vanadate (1 mM; 4 hours) and stimulated with Oncostatin M (40 ng/mL; 5 minutes) (positive). Whole-cell lysates were prepared and added to MULTI-SPOT 4 Spot plates coated with anti-total STAT3 antibody on one of the four spatially distinct electrodes per well. Phosphorylated pSTAT3 was detected with anti-phospho-STAT3 antibody labeled with MSD SULFO-TAG reagent.

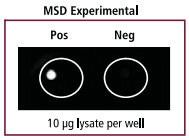
Phosphorylated STAT4 (Tyr693) Measured in T cells derived from Human PBMCs



Lysate (µg)	Positive			Negative			P/N
	Ave	StdDev	%CV	Ave	StdDev	%CV	
0	72	9	13	77	5	7	
0.63	1197	26	2	182	6	3	6.6
2.5	3963	122	3	263	5	2	15.1
10	10832	221	2	405	15	4	26.7

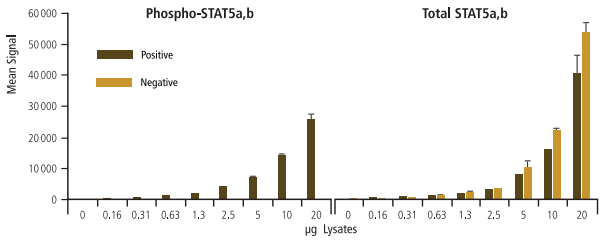




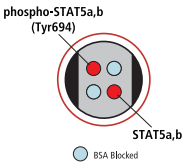


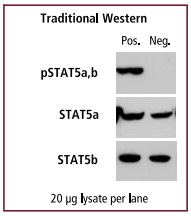
T cells were starved (30 minutes) (negative) and treated with IFNα (1000 U/mL) and IL-12 (10 ng/mL) for 30 minutes (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with anti-phospho-STAT4 antibody on one of the four spatially distinct electrodes per well. Phosphorylated STAT4 was detected with an anti-total STAT4 antibody labeled with MSD SULFO-TAG reagent.

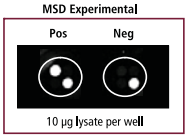
Simultaneous Quantification of Phosphorylated (Tyr694) and Total STAT5a,b in the Same Well



	Lysate (µg)	Positive			Negative			P/N
		Ave	StdDev	%CV	Ave	StdDev	%CV	
Phospho-STAT5a,b	0				89	7	8	
	0.16	288	16	6	66	3	4	4.4
	0.31	529	42	8	73	0	0	7.2
	0.63	959	61	6	71	19	27	13.6
	1.3	1867	44	2	87	8	9	21.6
	2.5	4021	35	1	68	6	9	59.6
	5	7287	141	2	114	4	4	63.9
	10	14256	196	1	132	1	1	108.0
	20	25753	1615	6	171	14	8	150.6
Total STAT5a,b	0				349	46	13	
	0.16	457	32	7	476	36	8	1.0
	0.31	660	6	1	681	0	0	1.0
	0.63	998	5	0	1244	89	7	0.8
	1.3	1712	148	9	2407	99	4	0.7
	2.5	2769	82	3	3522	14	0	0.8
	5	7545	419	6	10287	1925	19	0.7
	10	16159	120	1	22007	775	4	0.7
	20	40319	671	15	54094	3017	6	0.7

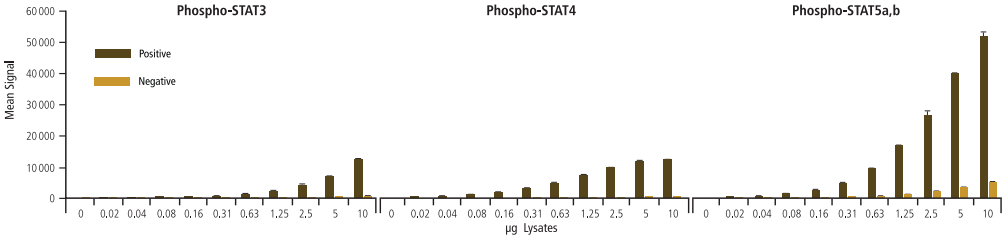


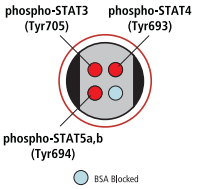




Confluent HeLa cells (negative) were pretreated with sodium vanadate (1 mM; 4 hours) and stimulated with Oncostatin M (40 ng/mL; 5 minutes) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with anti-phospho-STAT5a,b antibody and anti-total STAT5a,b antibody on two of the four spatially distinct electrodes per well. Phosphorylated and total STAT5a,b were detected with an anti-total STAT5a,b antibody labeled with MSD SULFO-TAG reagent.

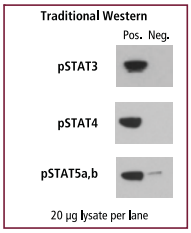
Multiple STAT Proteins are Detected Simultaneously in Human T Cells: Phospho-STAT3 (Tyr705), Phospho-STAT4 (Tyr693), and Phospho-STAT5a,b (Tyr694)





T cells were starved (30 minutes) (negative) and treated with IFNα (1000 U/mL) and IL-12 (10 ng/mL) for 30 minutes (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates coated with anti-total STAT3, anti-total STAT4, and anti-total STAT5a,b antibodies on three of the four spatially distinct electrodes per well. Phosphorylated STAT3, STAT4 and STAT5a,b were detected with anti-phospho-STAT3, anti-phospho-STAT4, and anti-phospho-STAT5a,b antibodies labeled with MSD SULFO-TAG reagent.

	Lysate (µg)	Positive			Negative			P/N
		Ave	StdDev	%CV	Ave	StdDev	%CV	
Phospho-STAT3	0				224	19	9	
	0.02	248	16	6	202	11	6	1.2
	0.04	294	6	2	206	2	1	1.4
	0.08	395	27	7	210	34	16	1.9
	0.16	551	16	3	203	20	10	2.7
	0.31	838	15	2	223	6	3	3.8
	0.63	1377	61	4	246	8	3	5.6
	1.25	2401	136	6	301	5	2	8.0
	2.5	4141	432	10	358	21	6	11.6
	5	7008	141	2	440	2	0	15.9
	10	12577	123	1	644	1	0	19.5
Phospho-STAT4	0				99	13	13	
	0.02	387	1	0	94	1	1	4.1
	0.04	666	38	6	101	11	11	6.6
	0.08	1154	59	5	116	6	6	10.0
	0.16	1982	62	3	110	10	9	18.0
	0.31	3223	53	2	147	7	5	21.9
	0.63	5113	84	2	186	8	4	27.6
	1.25	7419	221	3	256	20	8	29.0
	2.5	9573	282	3	322	1	0	29.8
	5	11788	382	3	410	5	1	28.8
	10	12550	10	0	499	0	0	25.2
Phospho-STAT5a,b	0				85	4	5	
	0.02	422	5	1	109	19	18	3.9
	0.04	798	43	5	118	10	8	6.8
	0.08	1533	22	1	148	18	12	10.4
	0.16	2825	67	2	256	7	3	11.0
	0.31	5102	16	0	401	8	2	12.7
	0.63	9440	172	2	684	5	1	13.8
	1.25	16708	392	2	1233	13	1	13.6
	2.5	26466	1423	5	2290	27	1	11.6
	5	40069	18	0	3465	69	2	11.6
	10	52072	1289	2	5227	29	1	10.0



Conclusions

- MSD manufactures multiplex assays that quantify various phosphoproteins in the Akt/mTOR and JAK/STAT pathways.
- Results obtained with MSD multiplex phosphoprotein assays are in agreement with conventional analysis by western blot with phospho-specific antibodies.
- Assays for the quantification of total protein are also available for many analytes.
- The assays are sensitive to sub-microgram levels of total protein input and compatible with lysates made from 384-well culture dishes.

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