High Throughput Assays for Biomarkers in 384-well Format

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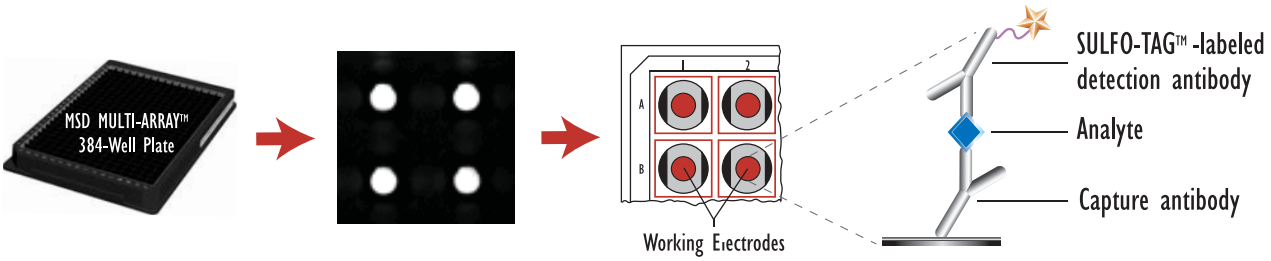
Monitoring biomarkers is common throughout the drug discovery process and increasingly popular in the earliest phases. Biomarker assays can be powerful readouts for cell based assays in support of focused library screening and the pursuit of structure-activity relationships. In order to maximally support these early drug discovery efforts, biomarker assays should be compatible with existing screening practices, ideally formatted in 384-well plates. We describe here a number of immunoassays for biomarkers formatted in 384-well plates. The assays are inclusive of a wide range of classes of biomarkers including intracellular phosphoproteins, the amyloid peptides of Alzheimer's Disease, and serum biomarkers. The assays are quantitative, sensitive, and retain the performance characteristics of the same assays in 96-well format. The assays are typically performed with 10 µL of sample making them well-suited to 384-well cell culture applications or the characterization of limiting amounts of fluids including cell supernatants, serum, plasma, and CSF.

Assay Protocol



General Protocol

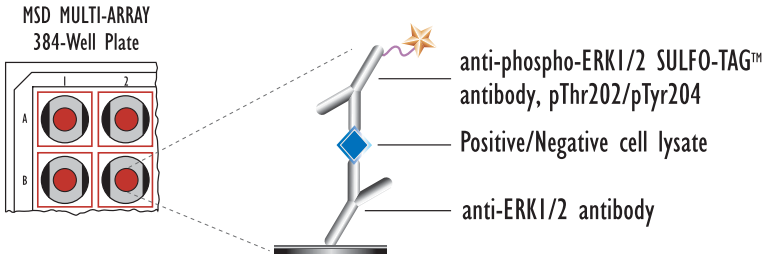
1. MSD MULTI-ARRAY 384-Well plates precoated with capture antibodies, plates are blocked and washed.

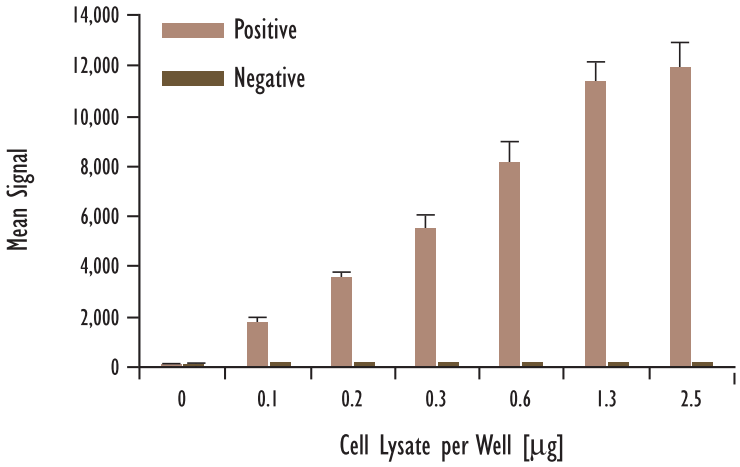
2. Samples are incubated in the assay plate with shaking, 10 µL per well, and then washed.

3. Antibodies labeled with MSD SULFO-TAG reagent are incubated in the assay plate with shaking, 10 µL per well, and then washed.

4. MSD Read Buffer T , 40 µL per well, followed by plate analysis on an MSD SECTOR Imager instrument.

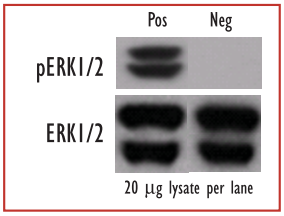
Detection of Phosphorylated ERK1/2 (pThr202/pTyr204)



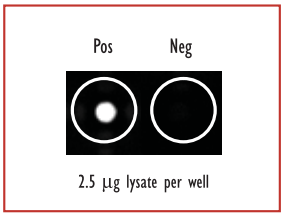


Lysates (µg)	Positive			Negative			P/N
	Average	StdDev	%CV	Average	StdDev	%CV	
0	116	13	11	108	13	12	
0.1	1,777	269	15	131	10	8	13.6
0.2	3,526	277	8	126	19	15	28.0
0.3	5,438	567	10	122	11	9	44.8
0.6	8,165	859	11	131	13	10	62.5
1.3	11,334	775	7	140	21	15	81.2
2.5	11,873	1,028	9	147	27	18	80.7

Traditional Western

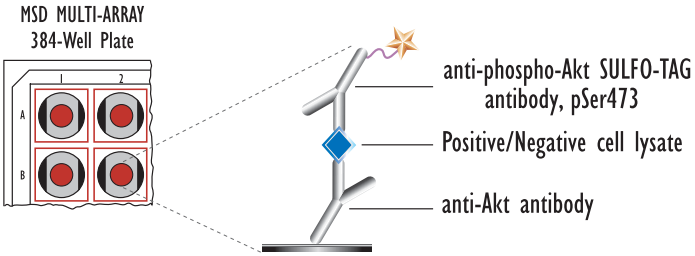


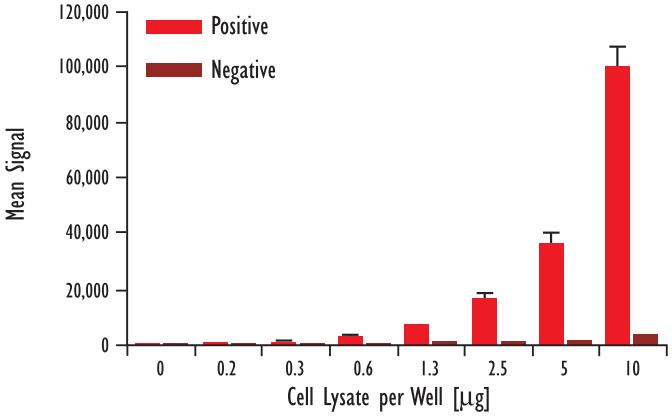
MSD Experimental



Jurkat cells were treated with PMA (200 nM; 15 minutes) (positive) or LY294002 (50 µM; 2.25 hr) (negative). Whole cell lysates were added to MSD MULTI-ARRAY 384-well plates coated with anti-ERK1/2 antibody. Phosphorylated ERK1/2 was detected with anti-phospho-ERK1/2 antibody labeled with MSD SULFO-TAG reagent.

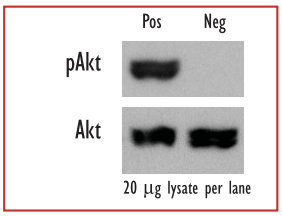
Detection of Phosphorylated Akt (pSer473)



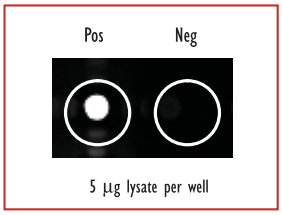


Lysates (µg)	Positive			Negative			P/N
	Average	StdDev	%CV	Average	StdDev	%CV	
0	65	10	15	103	8	7	
0.2	715	40	6	157	10	6	4.5
0.3	1,019	144	14	194	5	3	5.3
0.6	2,773	301	11	252	6	2	11.0
1.3	6,737	393	6	373	26	7	18.1
2.5	16,062	2,321	14	630	24	4	25.5
5	36,412	3,791	10	1,204	35	3	30.2
10	100,129	6,939	7	3,347	170	5	29.9

Traditional Western

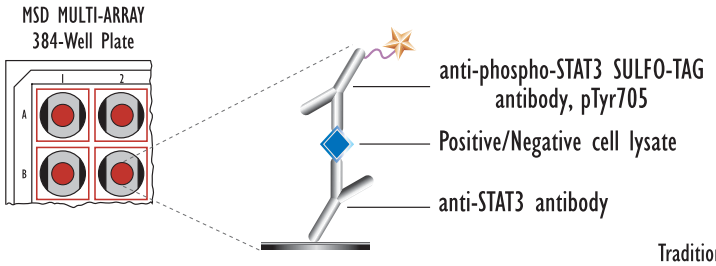


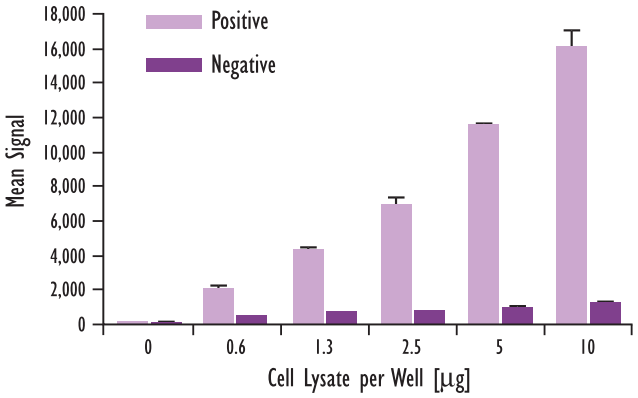
MSD Experimental



Logarithmically growing Jurkat cells (positive) were treated with LY294002 (50 µM; 2.25 hr) (negative). Whole cell lysates were added to MSD MULTI-ARRAY 384-well plates coated with an anti-total-Akt antibody. Phosphorylated Akt was detected with anti-phospho-Akt antibody labeled with MSD SULFO-TAG reagent.

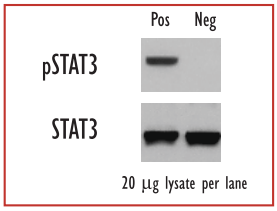
Detection of Phosphorylated STAT3 (pTyr705)



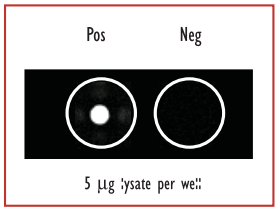


Lysates (µg)	Positive			Negative			P/N
	Average	StdDev	%CV	Average	StdDev	%CV	
0	133	10	8	119	8	6	
0.6	2,133	164	8	491	40	8	4.3
1.3	4,395	45	1	739	63	9	5.9
2.5	6,920	460	7	834	44	5	8.3
5	11,580	124	1	1,052	16	2	11.0
10	16,103	982	6	1,271	74	6	12.7

Traditional Western

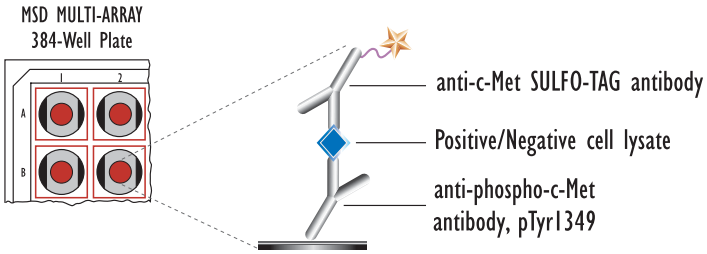


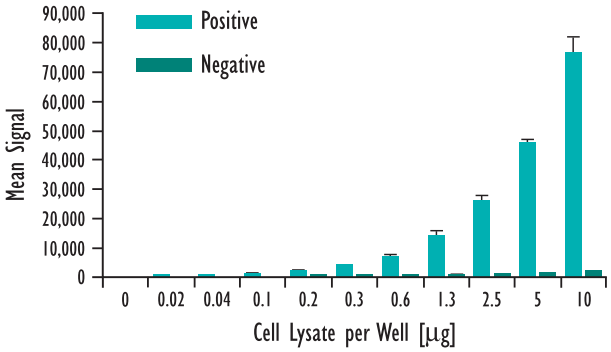
MSD Experimenta:



Confluent HeLa cells (negative) were pretreated with sodium vanadate (1mM, 4h) and stimulated with Oncostatin M (40ng/mL, 5min)(positive). Whole cell lysates were added to MSD MULTI-ARRAY 384-well plates coated with an anti-total-STAT3 antibody. Phosphorylated STAT3 was detected with anti-phospho-STAT3 antibody labeled with MSD SULFO-TAG reagent.

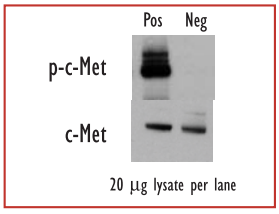
Detection of Phosphorylated c-Met (pTyr1349)



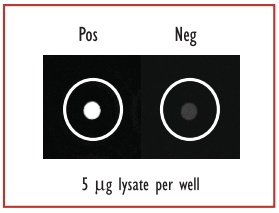


Lysates (µg)	Positive			Negative			P/N
	Average	StdDev	%CV	Average	StdDev	%CV	
0	172	6	3	151	8	6	
0.02	410	15	4	163	10	6	2.5
0.04	651	42	6	185	8	4	3.5
0.1	1,135	106	9	209	8	4	5.4
0.2	2,015	172	9	279	18	6	7.2
0.3	3,763	208	6	340	8	2	11.1
0.6	6,818	917	13	481	31	7	14.2
1.3	13,704	2,148	16	692	31	5	19.8
2.5	26,006	1,737	7	1,034	43	4	25.2
5	45,257	1,891	4	1,499	19	1	30.2
10	76,342	5,632	7	2,098	15	1	36.4

Traditiona: Western

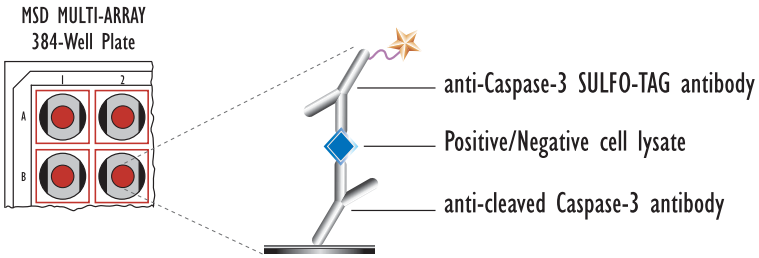


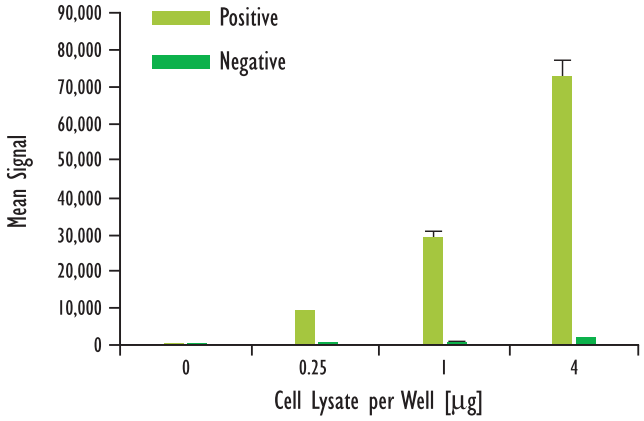
MSD Experimental



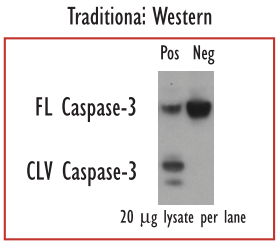
Growing confluent HeLa cells (negative) were treated with Sodium vanadate (1 mM) for 4 hr and then HGF (200 ng/mL) for 5 min (positive). Whole cell lysates were added to MSD MULTI-ARRAY 384-well plates coated with anti-phospho-c-Met antibody. Phosphorylated c-Met was detected with anti-c-Met antibody labeled with MSD SULFO-TAG reagent.

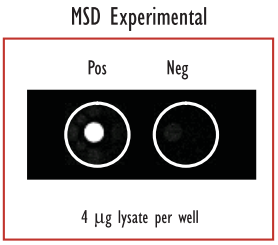
Detection of Cleaved, Active Caspase-3





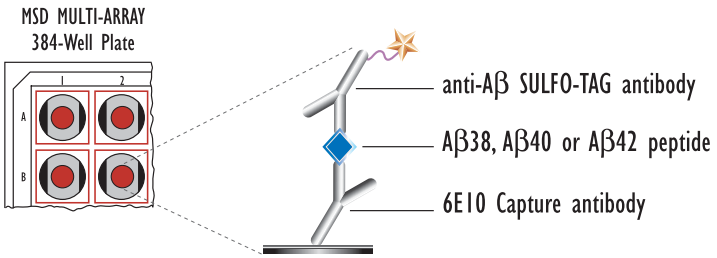
Lysates (µg)	Positive			Negative			P/N
	Average	StdDev	%CV	Average	StdDev	%CV	
0	54	8	14.9	40	5	11.6	
0.25	8,989	417	4.6	264	42	15.8	34.1
1	28,930	1,674	5.8	653	37	5.7	44.3
4	72,361	4,950	6.8	1,625	81	5.0	44.5



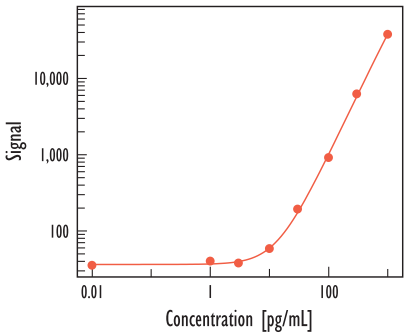


Logarithmically growing Jurkat cells (negative) were treated with staurosporine (1 µM; 4 hr)(positive). Whole cell lysates were added to MSD MULTI-ARRAY 384-well plates coated with anti-cleaved Caspase-3 antibody. Cleaved, active Caspase-3 was detected with an anti-Caspase-3 antibody labeled with MSD SULFO-TAG reagent.

Ultra-Sensitive Assays for Amyloid Peptides Aβ42, Aβ40, and Aβ38



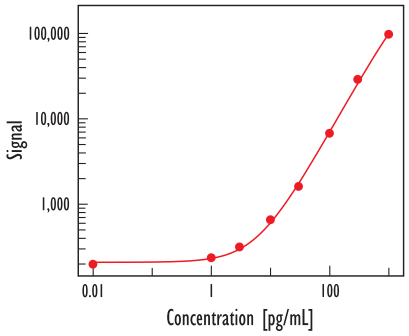
Aβ38



Peptide (pg/mL)	Aβ38			S/B
	Average	StdDev	%CV	
0	36	8	22	1
1	40	8	20	1
3	38	7	18	1
10	59	2	4	2
32	194	76	39	5
100	917	31	3	26
316	6,251	490	8	176
1,000	37,786	2,933	8	1,064

Aβ38 Lower Limit of Detection	10.1 pg/mL

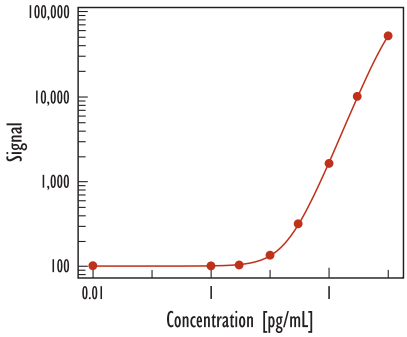
Aβ40



Peptide (pg/mL)	Aβ40			S/B
	Average	StdDev	%CV	
0	198	27	14	1
1	236	11	5	1
3	315	12	4	2
10	656	65	10	3
32	1,613	106	7	8
100	6,769	374	6	34
316	29,024	957	3	147
1,000	115,337	4,891	4	584

Aβ40 Lower Limit of Detection	2.1 pg/mL

Aβ42

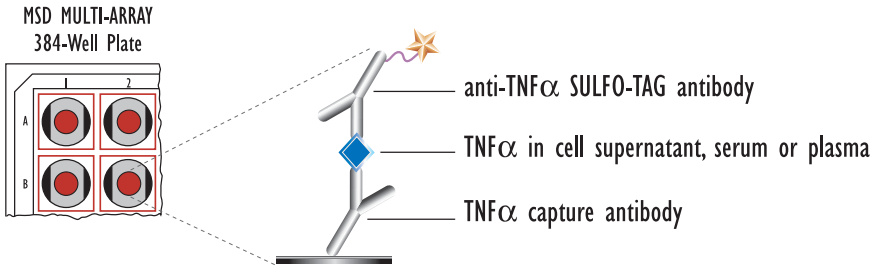


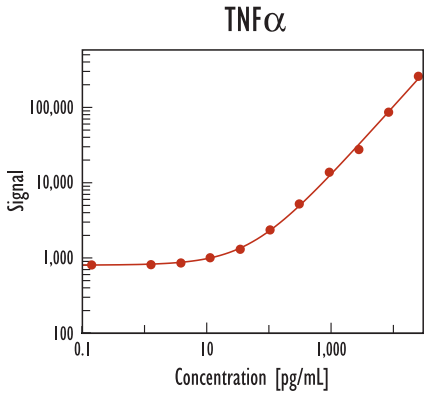
Peptide (pg/mL)	Aβ42			S/B
	Average	StdDev	%CV	
0	101	9	9	1
1	101	4	4	1
3	103	8	8	1
10	135	9	7	1
32	316	32	10	3
100	1,636	79	5	16
316	10,123	688	7	100
1,000	52,578	1,453	3	520

Aβ42 Lower Limit of Detection	8.7 pg/mL

The Aβ peptides are fragments of the amyloid precursor protein (APP) formed by sequential cleavage of APP by β-secretase and γ-secretase. One of the Aβ peptides, Aβ42, is the major component of amyloid plaques, the extracellular protein deposits characteristically seen in the brains of patients with Alzheimer's Disease (AD). MSD has developed novel antibodies to Aβ38, Aβ40, and Aβ42. Ultra-sensitive immuno-assays were developed for the amyloid peptides in combination with the 6E10 antibody that binds amino acids 3-8 near the N-terminus of the peptides. Here, synthetic peptides were diluted into a BSA solution and titrated in MSD MULTI-ARRAY 384-well plates coated with 6E10 antibody. The Aβ peptides were individually detected with MSD's peptide-specific antibodies labeled with SULFO-TAG.

Detection of Human Cytokine TNFα





TNFα Detection Limit:	7.6 pg/mL

TNFα regulates a wide range of cellular and systemic effects. It is one of the most commonly studied biomarkers in drug discovery. Quantitative measurements of TNFα are required for cell supernatants, serum and plasma. Here, recombinant, human TNFα had been diluted into tissue culture medium and titrated into MSD 384-well MULTI-ARRAY plates coated with an anti-TNFα capture antibody. In this homogenous assay, 10 µL of sample and 20 µL of anti-TNFα detection antibody in MSD Read Buffer are added sequentially to the well. The plate is incubated 4 hours and then analyzed in an MSD Sector Imager instrument.

Conclusions

- We have developed numerous immuno-assays in 384-well format for a wide range of biomarkers.
- The assays retain the performance of 96-well assays but require as little as 10 microliters of sample.
- The performance of the 384-well assays for intracellular phosphoproteins makes them compatible with screening applications using 384-well tissue cultures.
- Secreted proteins can be quantified from cell culture supernatants and animal fluids including serum, plasma, CSF and others.