A Suite of Assays to Detect Phosphorylated Receptor Tyrosine Kinases Associated with Neoplasia

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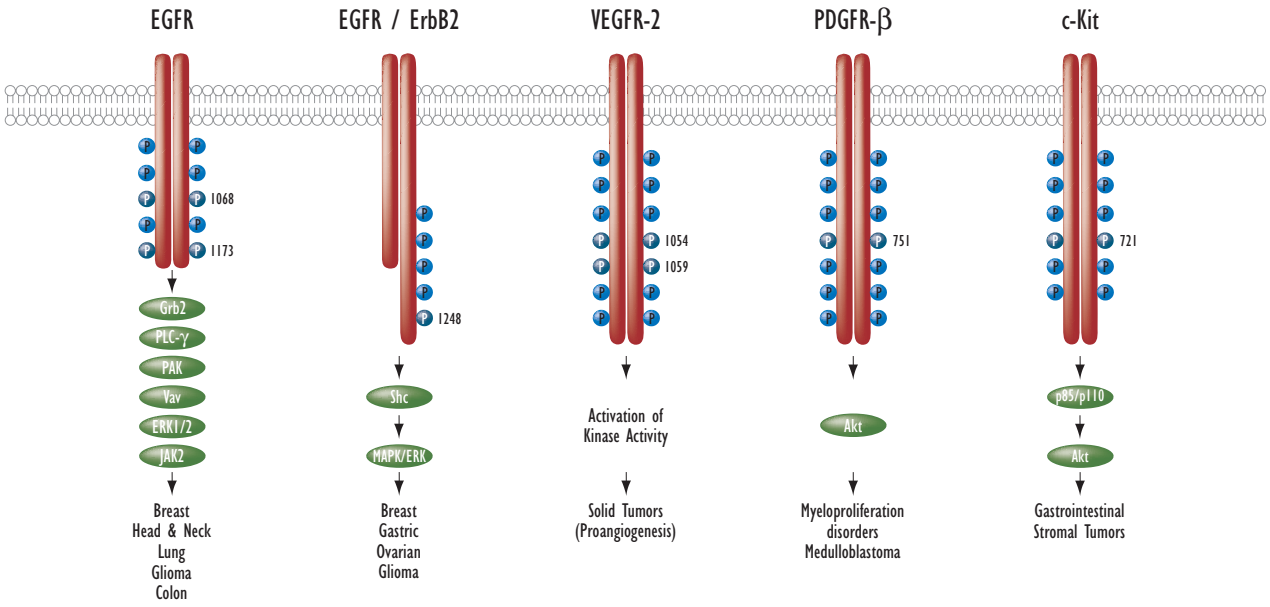
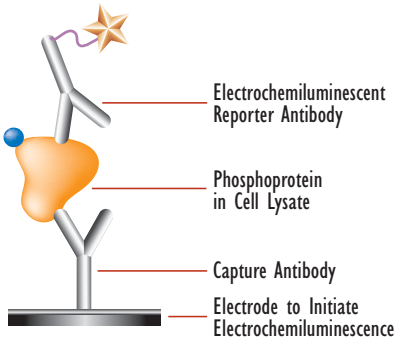
Reversible tyrosine phosphorylation is a critical process in the transduction of signals from the cell surface to the nucleus resulting in global changes in gene expression. A common hallmark of most neoplasia is the inappropriate expression or activation of cell surface proteins possessing inherent tyrosine kinase activity. These membrane spanning receptor tyrosine kinases (RTKs) phosphorylate themselves on tyrosine residues located on the cytoplasmic portion of the protein that can serve as docking sites for adapter proteins. The resulting multiprotein complexes form the framework for numerous signaling pathways involved in proliferation, differentiation, angiogenesis and cell survival. Here we describe a suite of individual and multiplex assays designed to assess the level of phosphorylation of EGFR, ErbB2, VEGFR-2, PDGFR-β and c-Kit in cellular lysates which can serve as surrogates to assess disease states. The assays are facile, sensitive and can be performed more rapidly than immunoblots and ELISAs and require considerably less material.

Five RTKs and Signaling Pathways Associated with Neoplasia



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

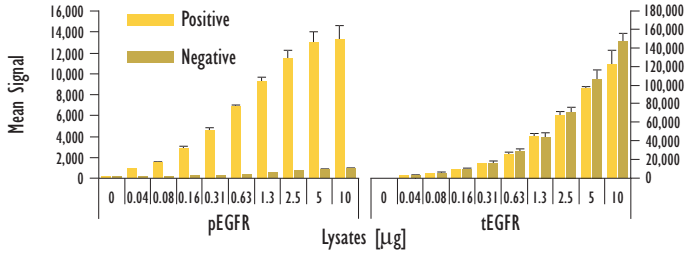
Assay Format

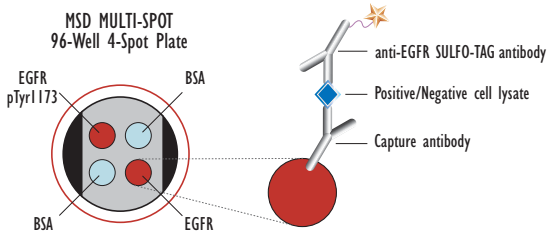


General Protocol

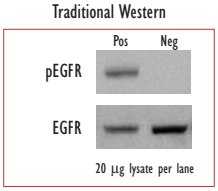
1. MULTI-SPOT 4 Spot 96-Well Plates precoated with capture antibodies are blocked with 150 µL of MSD Blocker A for 1 hr with shaking, followed by washing.
2. 25 µL of cell lysates are added to the wells and incubated for 1 hr with shaking, followed by washing.
3. 25 µL MSD SULFO-TAG™-labeled antibodies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

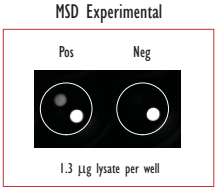
Detection of Phosphorylated and Total EGFR (pTyr1173) in the Same Well (A431 Cells)





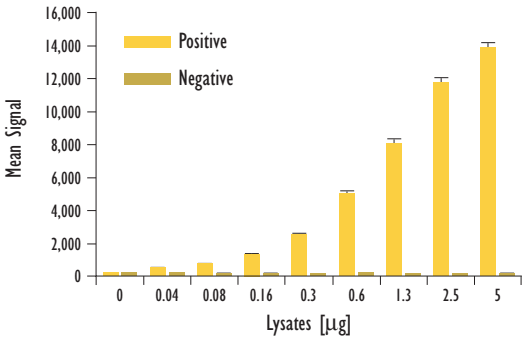
	Lysates (µg)	pEGFR Positive			pEGFR Negative			P/N
		Average	StdDev	%CV	Average	StdDev	%CV	
pEGFR	0	93	6	7	91	7	8	
	0.04	849	60	7	126	10	8	6.7
	0.08	1,523	95	6	116	5	4	13.2
	0.16	2,871	150	5	165	5	3	17.4
	0.31	4,560	261	6	211	19	9	21.7
	0.63	6,850	133	2	309	12	4	22.2
	1.3	9,301	383	4	460	27	6	20.2
	2.5	11,442	785	7	655	34	5	17.5
	5	12,953	1,077	8	821	39	5	15.8
	10	13,218	1,358	10	915	29	3	14.5
tEGFR	0	42	12	28	38	17	44	
	0.04	2,444	111	5	2,521	90	4	1.0
	0.08	4,491	255	6	4,227	878	21	1.1
	0.16	8,544	607	7	8,444	1,085	13	1.0
	0.31	15,328	816	5	15,130	2,323	15	1.0
	0.63	26,267	1,517	6	27,656	3,730	13	0.9
	1.3	44,901	3,633	8	43,875	4,322	10	1.0
	2.5	67,500	4,572	7	70,497	5,945	8	1.0
	5	95,616	2,311	2	106,279	10,174	10	0.9
	10	121,389	16,315	13	146,878	7,989	5	0.8



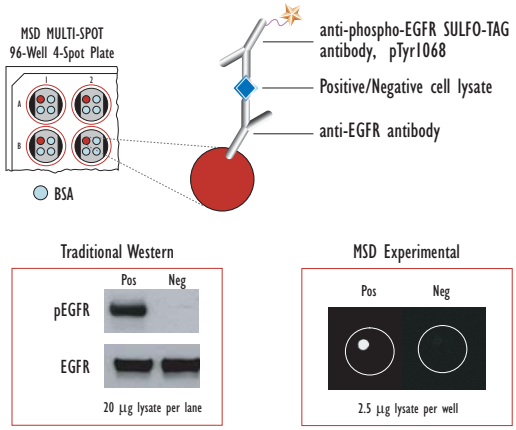


Serum-deprived A431 cells were treated with Compound 56 (1 µM; 2.5 hr)(negative) or EGF (100 ng/mL; 10 min)(positive). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with capture antibody on two of the four spatially distinct electrodes per well. Phosphorylated proteins were detected with MSD SULFO-TAG-labeled detection antibodies.

Detection of Phosphorylated EGFR (pTyr1068)

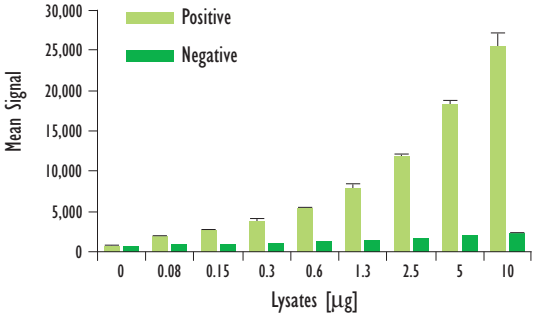


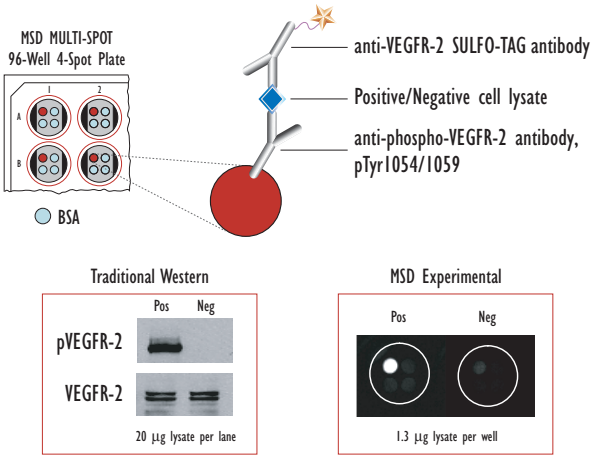
Lysates (µg)	pEGFR Positive			pEGFR Negative			P/N
	Average	StdDev	%CV	Average	StdDev	%CV	
0	192	14	7	184	15	8	
0.04	494	45	9	182	10	5	2.7
0.08	723	61	8	170	28	16	4.3
0.16	1,345	28	2	171	7	4	7.9
0.3	2,499	95	4	150	14	9	16.7
0.6	4,977	184	4	173	16	9	28.8
1.3	8,058	309	4	143	13	9	56.3
2.5	11,776	265	2	148	13	9	79.6
5	13,808	378	3	168	16	10	82.2



Serum-deprived A431 cells were treated with Compound 56 (1 µM; 2.5 hr) (negative) or EGF (100 ng/mL; 10 min)(positive). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-total-EGFR antibody on one of the four spatially distinct electrodes per well. Phosphorylated EGFR was detected with anti-phospho-EGFR antibody labeled with MSD SULFO-TAG reagent.

Detection of Phosphorylated VEGFR-2 (pTyr1054/1059)

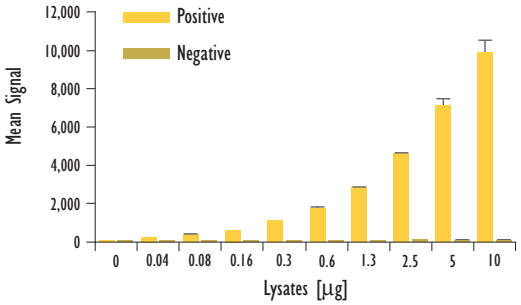




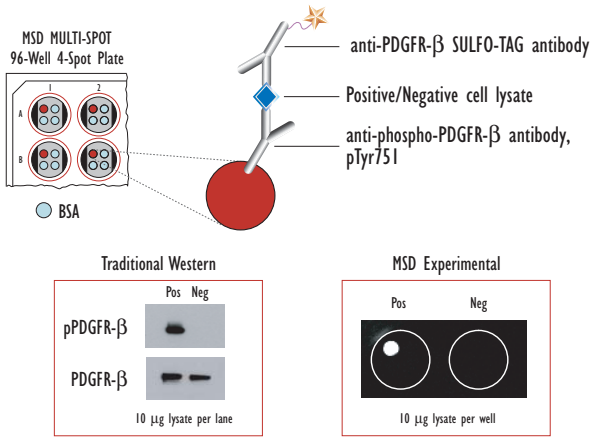
Lysates (µg)	pVEGFR-2 Positive			pVEGFR-2 Negative			P/N
	Average	StdDev	%CV	Average	StdDev	%CV	
0	637	64	10	591	61	10	
0.08	1,803	150	8	719	24	3	2.5
0.15	2,636	170	6	806	39	5	3.3
0.3	3,664	370	10	976	22	2	3.8
0.6	5,317	251	5	1,188	65	5	4.5
1.3	7,806	632	8	1,398	14	1	5.6
2.5	11,831	286	2	1,583	62	4	7.5
5	18,197	608	3	1,918	61	3	9.5
10	25,573	1,704	7	2,130	130	6	12.0

Logarithmically growing HEK293 cells expressing VEGFR-2 (negative) were treated with VEGF (5 min; 1 nM)(positive). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-VEGFR-2 antibody on one of the four spatially distinct electrodes per well. Phosphorylated VEGFR-2 was detected with anti-VEGFR-2 antibody labeled with MSD SULFO-TAG reagent.

Detection of Phosphorylated PDGFR-β

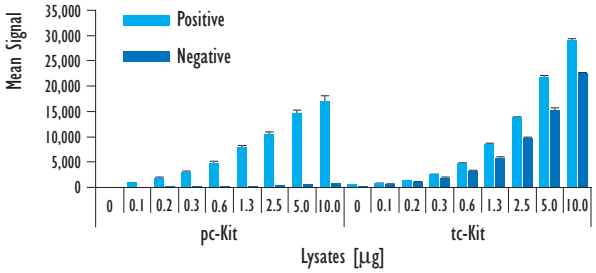


Lysates (µg)	pPDGFR-β Positive			pPDGFR-β Negative			P/N
	Average	StdDev	%CV	Average	StdDev	%CV	
0	65	9	13	72	11	16	
0.04	239	5	2	83	8	10	2.9
0.08	386	18	5	67	6	9	5.8
0.16	617	13	2	62	4	6	10.0
0.3	1,076	46	4	67	5	7	16.1
0.6	1,737	78	5	75	6	8	23.2
1.3	2,837	64	2	84	5	6	33.8
2.5	4,615	41	1	95	6	6	48.6
5	7,110	344	5	90	5	6	79.0
10	9,870	665	7	91	8	8	108.5

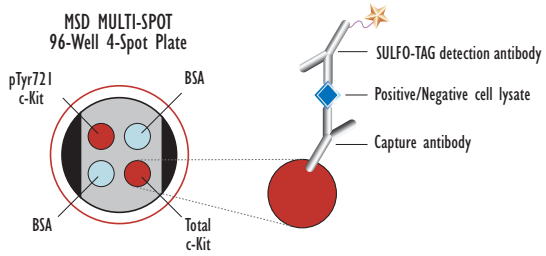


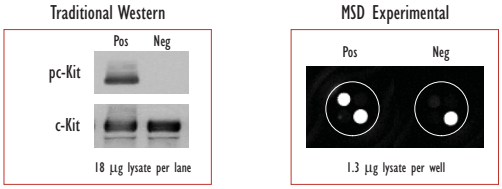
Serum-deprived NIH3T3 cells (negative) were treated with PDGF-BB (25 ng/mL; 10 min)(positive). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-phospho-PDGFR-β antibody on one of the four spatially distinct electrodes per well. Phosphorylated PDGFR-β was detected with anti-PDGFR-β antibody labeled with MSD SULFO-TAG reagent.

Detection of Phosphorylated and Total c-Kit (pTyr721) in the Same Well



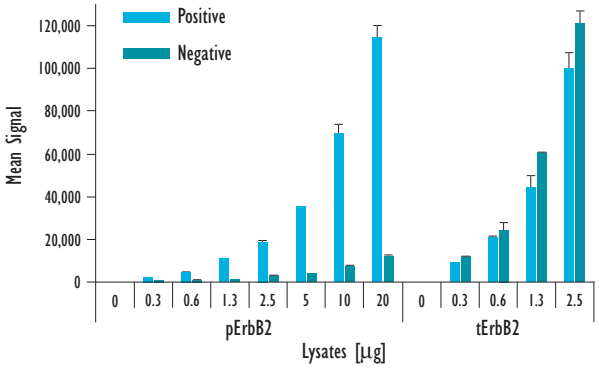
	Lysates (µg)	pc-Kit Positive			pc-Kit Negative			P/N
		Average	StdDev	%CV	Average	StdDev	%CV	
pc-Kit	0	36	7	20	37	7	19	
	0.1	999	87	9	52	7	13	19.2
	0.2	1,769	318	18	110	3	2	16.1
	0.3	3,008	284	9	140	5	3	21.4
	0.6	4,742	497	10	229	14	6	20.7
	1.3	7,823	445	6	305	10	3	25.6
	2.5	10,501	420	4	435	18	4	24.1
	5.0	14,574	774	5	583	75	13	25.0
	10.0	17,098	968	6	723	34	5	23.6
tc-Kit	0	568	37	7	304	15	5	
	0.1	799	37	5	665	57	9	1.2
	0.2	1,413	61	4	1,065	154	14	1.3
	0.3	2,481	156	6	1,794	230	13	1.4
	0.6	4,700	146	3	3,203	154	5	1.5
	1.3	8,386	366	4	5,755	287	5	1.5
	2.5	13,818	194	1	9,645	218	2	1.4
	5.0	21,765	271	1	15,144	583	4	1.4
	10.0	29,009	328	1	22,478	260	1	1.3



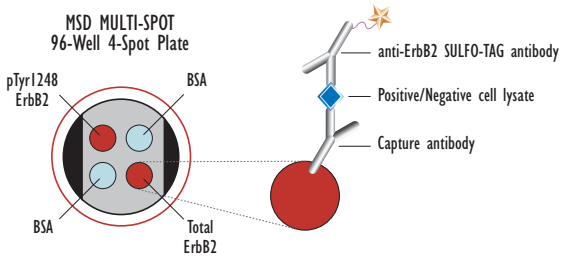


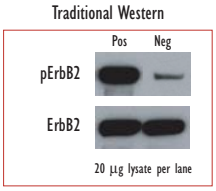
Growing MO7e cells were starved in low serum for 30 min (negative) and treated with SCF (100 ng/mL; 2 min)(positive). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-phospho-c-Kit and anti-total-c-Kit antibodies on two of the four spatially distinct electrodes per well. Phosphorylated and total c-Kit were detected with anti-total-c-Kit antibody labeled with MSD SULFO-TAG reagent.

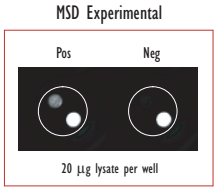
Detection of Phosphorylated and Total ErbB2 in the Same Well



	Lysates (µg)	pErbB2 Positive			pErbB2 Negative			P/N
		Average	StdDev	%CV	Average	StdDev	%CV	
pErbB2	0	143	46	32	103	16	15	
	0.3	2,106	177	8	453	15	3	4.7
	0.6	4,325	420	10	699	87	12	6.2
	1.3	10,257	788	8	1,132	97	9	9.1
	2.5	18,123	1,404	8	2,472	432	17	7.3
	5	34,926	434	1	3,465	39	1	10.1
	10	69,222	4,781	7	6,945	1,071	15	10.0
	20	114,128	5,981	5	11,531	1,180	10	9.9
tErbB2	0	143	46	32	103	16	15	
	0.3	8,778	370	4	11,393	412	4	0.8
	0.6	20,624	730	4	24,101	3,479	14	0.9
	1.3	44,007	5,903	13	60,051	596	1	0.7
	2.5	99,453	8,015	8	120,799	5,893	5	0.8
	5	184,498	7,473	4	216,863	4,572	2	0.9
	10	300,929	7,352	2	340,928	8,338	2	0.9
	20	417,262	10,865	3	451,655	10,079	2	0.9







Serum deprived SK-OV3 cells were treated with orthovanadate (1 mM; 5 hr) followed by EGF stimulation (100 ng/mL; 10 min)(positive) or Compound 56 (1 µM; 3 hr) and AG825 (1 µM; 3 hr)(negative). Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-phospho-ErbB2 antibody and anti-total-ErbB2 antibody on two of the four spatially distinct electrodes per well. Phosphorylated and total ErbB2 were detected with anti-total-ErbB2 antibody labeled with MSD SULFO-TAG reagent.

Determining % Phosphoprotein: Phosphorylated and Total Assay in the Same Well

- MSD phosphorylated/total multiplex assays have been optimized to account for differences in the binding affinity between the phosphorylated and total antibodies.
- The capture antibodies may differ in their weak binding to abundant proteins in the test lysate, therefore each assay in the well may not be linear over the same concentration range.
- Different protein targets may vary greatly in abundance in a particular sample, thus the establishment of a linear assay range for each target is recommended.

	pErbB2		tErbB2	
Lysates (µg)	Positive Signal	Negative Signal	Positive Signal	Negative Signal
0	143	103	143	103
0.3	2,106	453	8,778	11,393
0.6	4,325	699	20,624	24,101
1.3	10,257	1,132	44,007	60,051
2.5	18,123	2,472	99,453	120,799
5	34,926	3,465	184,498	216,863
10	69,222	6,945	300,929	340,928
20	114,128	11,531	417,262	451,655

Lysates (µg)	% pErbB2 Positive	% pErbB2 Negative
0		
0.3	38.7	7.6
0.6	34.7	4.9
1.3	37.8	3.7
2.5	30.8	4.0
5	31.8	3.1
10	37.4	4.0
20	43.0	5.0

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 + total" since the actual "total" is all of the material detected on both spots.

Conclusions

- We describe a suite of assays to detect the phosphorylation state of five receptor tyrosine kinases implicated in neoplasia.
- The multiplex configuration of the phosphorylated and total assays in the same well allows for quantifying the amount of both pools of receptor within the same well reducing the amount of sample consumed per data point.
- An estimate of the percentage of receptor that is phosphorylated can be determined since both forms of protein are detected within a single cell.
- The assays offer a high throughput alternative to conventional immunoblots for determining if a protein is post-translationally modified by phosphorylation at a specific amino acid(s).
- The assays are rapid and sensitive with detection limits in the submicrogram range of total cell lysate, resulting in assays that are compatible with 96-well and, in certain cases, 384-well cultures.