Abstract #5835

Development of a Multiplex Screening Panel for Akt Signaling Pathway Biomarkers in Cell and Tissue Lysate Models

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1 Abstract

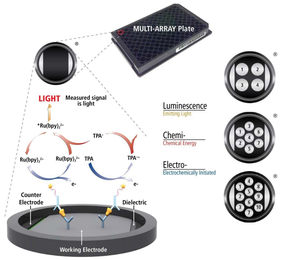
Major challenges face the large-scale implementation of intracellular tumor biomarkers for clinical diagnosis and therapeutic development. These challenges include non-quantitative results, insufficient assay sensitivity, and lack of multiplexing capability. For many studies, sample volumes are limited (e.g., tumor lysates); however, it is essential to extract as much biomarker information as possible from a given sample while maintaining the quality and consistency necessary to sustain ongoing studies. To address these challenges, MSD® has developed multiplex panels for assaying cell signaling biomarkers using fit-for-purpose methods that emphasize optimal multiplex combinations and rigorous development of critical reagents. Here we report the development and verification of a multiplex screening panel of immunoassays for simultaneous measurement of total and phosphorylated analytes of the Akt signaling pathway using MSD technology. Markers in the panel include phosphorylated and total forms of GSK3B, p70S6K, FOXO3a, PTEN, Akt, S6RP, PRAS40, and ERK1.

We demonstrate the ability of these assays to measure analyte levels in multiple tumor-derived cell lines and human tissue samples (normal and tumor) with excellent sensitivity and performance. Most analytes can be quantified using no more than 10 µg of sample. By using recombinant proteins to calibrate some of the assays, we were able to quantify analytes in cultured cell lysates, including MCF-7 and Jurkat T-cells. Lack of analyte specificity is a well-known issue when multiplexing intracellular signaling analytes, especially those in a common signaling pathway such as the Akt pathway. Through rigorous optimization, we achieved non-specific binding less than or equal to 2% for all analytes in this panel. Spike recovery and dilution linearity were matrix tolerant in tumor cell line and tissue lysates. In addition, the assays exhibited a dynamic range between 3 and 4 logs. This characterization allowed for quantification of analytes at different abundance levels without using multiple dilutions.

In conclusion, MSD MULTI-SPOT® Akt signaling assay panels have been developed and verified for measurement of intracellular tumor biomarkers of relevance to clinical diagnosis, therapeutic development, and treatment of various cancers. The accuracy, reliability, ease of use, and high-throughput features of these multiplex assays make them ideally suited for use in large-scale clinical studies.

2 Methods

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. The multiplex panels were developed as two 8-plex panels designed for optimal performance. The assays use a common Total-analyte specific capture antibody and an assay specific total or phospho-specific detection antibody. Assay controls were prepared by diluting blended Jurkat and MCF7 cell lysates to 400 µg/mL, 100 µg/mL, and 25 µg/mL in lysis buffer supplemented with protease and phosphatase inhibitors. We performed a complete analytical validation of the kits.



Electrochemiluminescence Technology

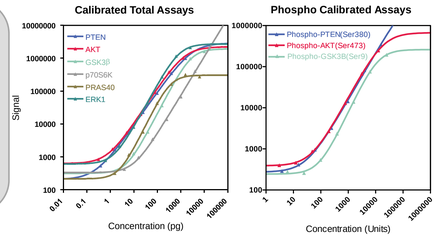
- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

3 Protocol and Calibration Curves

The panels use a simple 4-step protocol that can be completed in 3-3.5 hours. Representative calibration curves are shown below for each of the 9 Calibrated assays. While only calibrators for the 9 specified assays were available at the time this study was completed, MSD is continuing to develop calibrators for the remaining assays.

Protocol

1. Add 150 µL Blocker A. Incubate for 1 hour at room temperature (RT).
2. Wash with TBS-T. Add 25 µL of calibrator, control, or diluted sample. Incubate for 1 hour at RT.
3. Wash with TBS-T. Add 25 µL of detection antibody. Incubate for 1 hour at RT.
4. Wash with TBS-T. Add 150 µL of 1X Read Buffer T. Read on MSD imager.



4 Sensitivity

The lower limit of detection (LLOD) is a calculated concentration corresponding to the signal 2.5 standard deviations above the background (zero calibrator).

	GSK3B			p70S6K			PTEN		
	Conc., (pg)	Average Signal	%CV	Conc., (pg)	Average Signal	%CV	Conc., (pg)	Average Signal	%CV
Cal-1	10000.00	1596097	9.6	16000.00	2059368	0.2	1562.50	988802	8.3
Cal-2	2500.00	971158	14.5	4000.00	401055	5.0	390.63	348648	6.8
Cal-3	625.00	225626	1.6	1000.00	67571	6.9	97.66	86694	0.7
Cal-4	156.25	36455	8.4	250.00	13887	3.6	24.41	22713	0.8
Cal-5	39.06	5728	8.5	62.50	3445	5.5	6.10	5632	9.0
Cal-6	9.77	1265	11.9	15.63	966	4.1	1.53	1641	1.2
Cal-7	2.44	432	12.3	3.91	422	8.9	0.38	536	10.4
Cal-8	0	144	6.9	0	234	10.0	0	198	0.0
	1.25	**LLOD**		**2.50**	**LLOD**		**0.09**	**LLOD**	

	AKT			PRAS40			ERK1		
	Conc., (pg)	Average Signal	%CV	Conc., (pg)	Average Signal	%CV	Conc., (pg)	Average Signal	%CV
Cal-1	1250.00	1024694	3.9	6250.00	274312	26.8	2500.00	2116114	0.7
Cal-2	312.50	380428	4.6	1562.50	319782	6.4	625.00	1155337	4.1
Cal-3	78.13	92211	0.1	390.63	163216	2.3	156.25	270121	0.7
Cal-4	19.53	19866	3.1	97.66	42839	2.1	39.06	44137	4.6
Cal-5	4.88	5411	2.8	24.41	5702	16.3	9.77	8059	0.9
Cal-6	1.22	1727	5.4	6.10	1164	1.2	2.44	2101	2.8
Cal-7	0.31	838	2.6	1.53	317	8.5	0.61	785	4.1
Cal-8	0	531	0.5	0	154	12.9	0	426	8.5
	0.10	**LLOD**		**1.02**	**LLOD**		**0.27**	**LLOD**	

	Phospho-GSK3B(Ser9)			Phospho-PTEN(Ser380)			Phospho-AKT(Ser473)		
	Conc., (Units)	Average Signal	%CV	Conc., (Units)	Average Signal	%CV	Conc., (Units)	Average Signal	%CV
Cal-1	100000	208121	10.0	15625	314200	4.9	12500	150284	7.1
Cal-2	25000	61834	6.1	3906	67487	4.7	3125	35749	5.5
Cal-3	6250	8955	3.3	977	11238	5.4	781	5835	2.9
Cal-4	1563	1319	5.2	244	2568	3.0	195	1453	4.1
Cal-5	391	348	8.4	61	806	6.2	49	754	2.7
Cal-6	98	199	7.0	15	405	4.9	12	537	6.0
Cal-7	24	162	28.6	4	329	4.5	3	508	7.5
Cal-8	6	152	21.4	1.0	377	17.1	0.8	473	7.0
	277	**LLOD**		**16.1**	**LLOD**		**29**	**LLOD**	

5 Specificity and Reproducibility

Each assay in the 16-plex panel was evaluated for cross-reactivity with other analytes on the panel as well as related analytes that are not on the panel. The cross-reactivity for all assays was <2 %.

To evaluate reproducibility, three levels of controls spanning the quantifiable range of the panels were prepared and measured over multiple runs. The %CV for each assay of the 16-plex assays is shown below. The controls were prepared for single-use, and 7 runs were conducted.

CTL-1	Non-Specific Interaction	Total Detect							
		PTEN	*Akt*	GSK3b	FOXO3a	PRAS40	ERK1	p70S6K	S6RP
Capture	PTEN	100.00%	0.00%	0.02%	0.01%	0.05%	0.02%	0.01%	<0.1%
	Akt	0.06%	100.00%	0.15%	0.01%	0.01%	0.07%	0.02%	<0.1%
	GSK3b	0.08%	<0.1%	100.00%	0.02%	0.00%	<0.1%	0.14%	0.73%
	FOXO3a	0.08%	0.02%	0.01%	100.00%	0.04%	0.01%	0.49%	0.31%
	PRAS40	0.09%	<0.1%	0.00%	0.42%	100.00%	0.00%	0.00%	0.02%
	ERK1	0.03%	0.04%	0.01%	0.01%	0.04%	100.00%	0.01%	0.08%
	p70S6K	<0.1%	<0.1%	0.00%	0.00%	<0.1%	0.01%	100.00%	<0.1%
	S6RP	0.08%	0.04%	0.01%	0.05%	0.00%	0.01%	0.32%	100.00%

CTL-1	Non-Specific Interaction	Phospho Detect							
		PTEN	*Akt*	GSK3b	FOXO3a	PRAS40	ERK1	p70S6K	S6RP
Capture	PTEN	100.00%	0.00%	0.02%	0.36%	0.03%	0.14%	0.00%	0.04%
	Akt	0.09%	100.00%	0.19%	0.05%	0.00%	0.27%	0.00%	0.03%
	GSK3b	0.65%	<0.1%	100.00%	1.17%	0.00%	0.17%	0.00%	1.04%
	FOXO3a	1.02%	0.02%	0.00%	100.00%	0.00%	0.16%	0.00%	1.21%
	PRAS40	1.34%	<0.1%	<0.1%	0.05%	100.00%	0.01%	0.00%	0.01%
	ERK1	0.14%	1.18%	0.00%	0.09%	0.02%	100.00%	0.00%	0.09%
	p70S6K	0.02%	0.02%	0.01%	0.01%	0.00%	<0.1%	100.00%	0.01%
	S6RP	1.28%	0.04%	0.04%	1.91%	0.01%	<0.1%	0.00%	100.00%

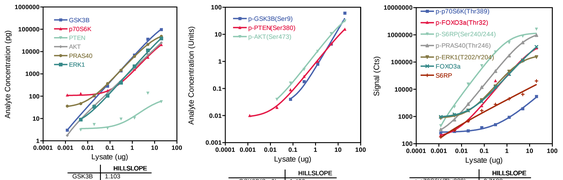
Assay	Sample ID	AVG Intra-run Signal CV	AVG Intra-run Conc CV
GSK3B	Ctl-1	6.6%	5.7%
	Ctl-2	5.5%	4.4%
	Ctl-3	5.1%	4.2%
p70S6K	Ctl-1	2.6%	2.4%
	Ctl-2	1.6%	1.5%
	Ctl-3	3.7%	3.5%
FOXO3a	Ctl-1	2.8%	N/A
	Ctl-2	4.1%	N/A
	Ctl-3	4.0%	N/A
PTEN	Ctl-1	0.8%	0.8%
	Ctl-2	4.6%	4.6%
	Ctl-3	4.4%	4.6%
AKT	Ctl-1	4.4%	3.9%
	Ctl-2	7.2%	6.3%
	Ctl-3	4.6%	4.5%
S6RP	Ctl-1	1.3%	N/A
	Ctl-2	1.7%	N/A
	Ctl-3	4.4%	N/A
PRAS40	Ctl-1	3.9%	3.5%
	Ctl-2	6.0%	4.9%
	Ctl-3	2.2%	1.8%
ERK1	Ctl-1	4.8%	4.5%
	Ctl-2	3.8%	3.1%
	Ctl-3	2.2%	1.8%

Assay	Sample ID	AVG Intra-run Signal CV	AVG Intra-run Conc CV
Phospho-GSK3B(Ser9)	Ctl-1	7.1%	23.0%
	Ctl-2	7.1%	6.2%
	Ctl-3	5.8%	4.1%
Phospho-p70S6K(Thr389)	Ctl-1	5.7%	11.7%
	Ctl-2	2.7%	6.0%
	Ctl-3	1.5%	4.4%
Phospho-FOXO3a(Thr32)	Ctl-1	5.4%	N/A
	Ctl-2	8.8%	N/A
	Ctl-3	6.5%	N/A
Phospho-PTEN(Ser380)	Ctl-1	6.1%	5.2%
	Ctl-2	4.0%	3.5%
	Ctl-3	3.7%	3.4%
Phospho-AKT(Ser473)	Ctl-1	2.7%	2.6%
	Ctl-2	4.3%	3.6%
	Ctl-3	3.7%	3.3%
Phospho-S6RP(Ser240/244)	Ctl-1	3.1%	N/A
	Ctl-2	3.4%	N/A
	Ctl-3	1.0%	N/A
Phospho-PRAS40(Thr246)	Ctl-1	4.1%	N/A
	Ctl-2	14.7%	N/A
	Ctl-3	11.7%	N/A
Phospho-ERK1(T202/Y204; T185/Y187)	Ctl-1	9.8%	N/A
	Ctl-2	8.5%	N/A
	Ctl-3	8.5%	N/A

6 Spike Recovery and Dilution Linearity

Dilution linearity was assessed by serially diluting 20 µg/well (800 µg/mL) of different cell lysates. The calculated concentration or signal level of each analyte versus lysate concentration is shown along with calculated hillslopes. A hillslope of 1 for the dilution series is ideal.

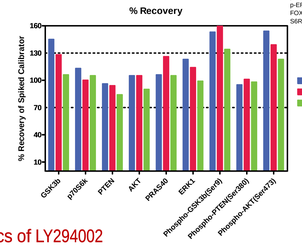
Spike recovery was assessed by diluting cell lysate samples to 0.63 µg/well (25 µg/mL) and spiking with calibrator at 3 levels throughout the assay range. Recoveries were within 30% of the expected concentrations with few exceptions. Dashed black lines represent boundaries of +/-30% recovery.



HILLSLOPE	
GSK3B	1.103
p70S6K	1.108
PTEN	0.9824
AKT	0.9395
PRAS40	1.074
ERK1	1.108

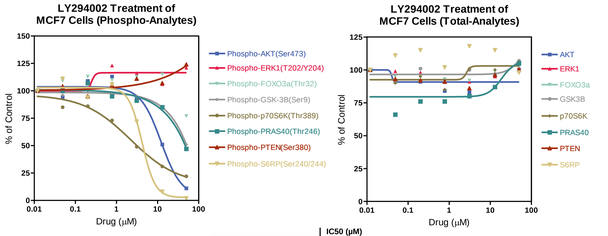
HILLSLOPE	
p-GSK3B(Ser9)	1.422
p-PTEN(Ser380)	1.043
p-AKT(Ser473)	1.075

HILLSLOPE	
p-p70S6K(Thr389)	0.7188
p-FOXO3a(Thr32)	1.017
p-S6RP(Ser240/244)	1.101
p-PRAS40(Thr246)	1.045
p-ERK1(T202/Y204)	1.036
FOXO3a	0.9214
S6RP	0.4312



7 Pharmacodynamics of LY294002

Human MCF7 cells were treated with serial dilutions of the PI3K inhibitor LY294002 for 30 minutes. ECL signals from treated samples were normalized to ECL signals in untreated samples. IC_{50} is the concentration that inhibited the indicated analyte phosphorylation by 50%. Total analyte levels were not affected by treatment. Analysis was performed using 5 µg/mL of lysate. No IC_{50} is reported for phospho-FOXO3a(Thr32) as the data could not be fit to a nonlinear regression curve.

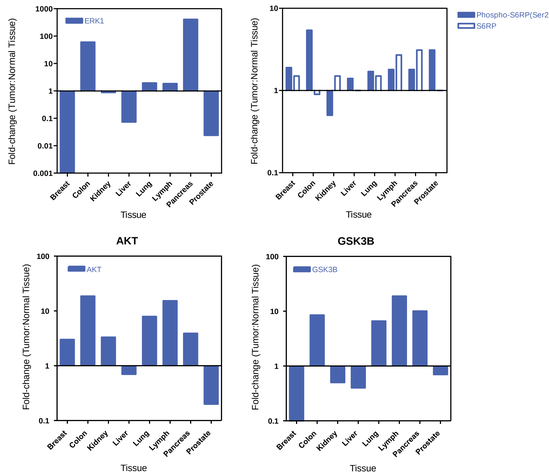


	IC50 (µM)
Phospho-AKT(Ser473)	12.17
Phospho-ERK1(T202/Y204)	--------
Phospho-FOXO3a(Thr32)	--------
Phospho-GSK-3B(Ser9)	53.24
Phospho-p70S6K(Thr389)	2.209
Phospho-PRAS40(Thr246)	46.82
Phospho-PTEN(Ser380)	--------
Phospho-S6RP(Ser240/244)	4.199

8 Quantitation in Human Tissue Samples

Total and phospho-analytes were measured in normal and tumor human tissue lysates (obtained from Novus Biologicals) from 5 µg of sample. Analyte levels varied across tissue types and normal/tumor status indicated by calculated concentration for calibrated assays and mean signal for non-calibrated assays in the table. ND indicates not detectible. Optimization of lysis conditions may improve analyte detection. Samples were not collected with optimal concentrations of phosphatase inhibitors. Graphs below are representative of analyte changes across tissues expressed as fold change in tumor to normal.

		AKT (pg)	Phospho-AKT (Ser473) (Units)	ERK1 (pg)	Phospho-ERK1 (T202/Y204) (cts)	GSK3B (pg)	Phospho-GSK3B (Ser9) (Units)	p70S6K (pg)	Phospho-p70S6K (Thr389) (cts)	PTEN (pg)	Phospho-PTEN (Ser380) (Units)	FOXO3a (cts)	Phospho-FOXO3a (Thr32) (cts)	S6RP (cts)	Phospho-S6RP (Ser240/244) (cts)
Normal	Breast	1.05	ND	587.12	ND	13.00	ND	3.07	ND	0.20	ND	ND	ND	4731	1103
	Colon	ND	ND	5.10	ND	3.04	ND	2.06	ND	0.09	ND	ND	ND	4740	1893
	Kidney	0.04	ND	98.48	ND	2.79	ND	1.60	ND	0.06	ND	ND	ND	4819	1758
	Liver	0.28	4.46	100.31	ND	1.82	47.72	1.43	ND	0.06	ND	ND	ND	4938	1874
	Lung	0.08	ND	82.09	ND	0.74	ND	1.60	ND	0.07	ND	ND	ND	2632	1233
	Lymph	0.21	ND	173.26	ND	0.46	ND	1.45	ND	0.08	ND	ND	ND	2861	1072
	Pancrea	0.01	1.22	0.08	ND	0.34	ND	1.05	ND	0.05	ND	ND	ND	3105	1178
	Prostate	2.79	3.56	335.18	ND	9.67	17.45	17.79	1286	0.36	ND	4856	ND	7772	973
Tumor	Breast	4.46	3.74	0.29	ND	1.66	ND	4.58	ND	0.43	ND	9235.00	ND	7072	2130
	Colon	10.25	18.27	308.82	5476.00	21.80	257.33	8.98	764.00	2.75	ND	5357.00	ND	4074	10283
	Kidney	1.41	ND	86.87	ND	1.18	ND	2.01	ND	0.11	27.98	ND	ND	7151	909
	Liver	0.06	ND	7.37	ND	0.48	ND	1.73	ND	0.07	ND	ND	ND	4708	2687
	Lung	4.63	ND	157.14	3569.00	6.64	ND	2.29	ND	0.17	ND	ND	ND	3852	2050
	Lymph	11.98	21.31	317.60	ND	13.95	66.71	21.38	1495.00	0.80	14.18	7801.00	1333.00	7658	1930
	Pancrea	1.68	5.53	255.20	ND	7.19	161.03	2.66	ND	0.19	ND	ND	ND	9594	2142
	Prostate	0.05	ND	8.37	ND	6.68	ND	2.12	ND	0.10	ND	4007	1868.00	7550	2973



9 Conclusion

A multiplex screening panel has been developed and validated for use in quantitating Akt signaling analytes in a variety of human samples. The assays in the panel have sufficient sensitivity, reproducibility, accuracy, and sample performance to allow testing of multiple sample types in cancer biology applications. Consistent results were demonstrated for calibration curves as well as for control samples over multiple runs on different days by different operators. All of the assays in the panel can be run using two fit-for-purpose-designed 8-plex panels that use a simple three-step protocol. Biomarker screening can be performed with as little as 5 µg of sample. Evaluation of various disease samples using this panel demonstrates that it can be an effective tool for cancer biomarker and drug discovery.

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