Exploring Akt/mTOR Signaling
Using the MILLIPLEX® MAP Akt/mTOR 11-plex Panel

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Dr. Hwang develops new MILLIPLEX MAP MAPmates™ signaling assays and kits for the Luminex® xMAP® bead-based platform. His extensive experience in cell signaling research includes studies of insulin receptor processing and crosstalk between insulin signaling pathways. Dr. Hwang received his Ph.D. at the University of Florida, completed postdoctoral training at the University of Michigan, and developed multiple protein detection assays at LI-COR Biosciences before joining Merck Millipore. He has received numerous research awards and has authored or coauthored many peer-reviewed publications of his cell signaling research.

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Introduction

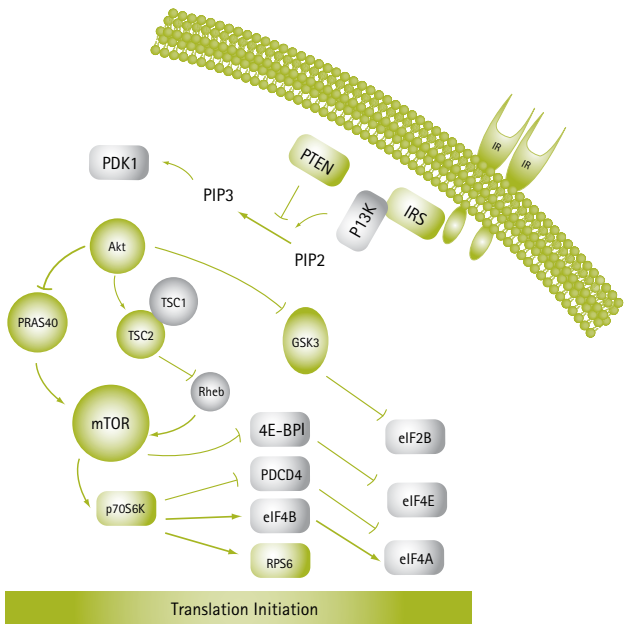
The Akt signaling pathway, one of the most often-dysregulated signaling pathways in cancer, plays an important role in mediating a very broad range of cellular processes such as growth and development, cell cycle, energy homeostasis, and survival. Akt, a serine/threonine kinase that phosphorylates over 100 protein substrates, mediating cell survival and proliferation signals, is often itself hyperphosphorylated in various tumor types. Although the Akt gene itself is not known to be mutated in cancers, many of the upstream regulators of Akt, including IR, IRS, PI3K and PTEN, are oncogenes and tumor suppressors. Downstream of Akt, the mammalian Target Of Rapamycin (mTOR) complex is a key regulator of growth and metabolism.

As nearly all of the players in the Akt/mTOR signaling pathway are coordinately regulated by phosphorylation, understanding the role of this pathway in normal physiological processes and in diseases such as cancer and diabetes requires the ability to simultaneously measure phosphorylation status of multiple protein targets.

Several assays to examine phosphorylation status are currently available, including Western blotting, ELISA, reverse phase arrays, quantitative cell imaging, and mass spectroscopy. Although some of these platforms yield absolute, quantitative data, the assays are either limited to measuring only one analyte at a time, or are excessively difficult or expensive.

Figure 1.
The Akt/mTOR signaling pathway. Analytes included in the MILLIPLEX MAP Akt/mTOR 11-plex Panel are highlighted in green.

MILLIPLEX MAP 11-plex Akt/mTOR Panel Analytes (phosphorylated residue(s))
Akt (Ser473)
GSK3α(Ser21)
GSK3β(Ser9)
IGF1R (Tyr1135/Tyr1136)
IR (Tyr1162/Tyr1163)
IRS1 (Ser312)
mTOR (Ser2448)
p70S6K (Thr424)
PTEN (Ser380)
RPS6 (Ser235/Ser236)
TSC2 (Ser939)



On the level of multiparametric single-cell analysis, flow cytometry has enabled the study of multiple pathway activation and cross-talk in a time-dependent manner. Directly applicable to the Akt/mTOR pathway, Merck Millipore's FlowCellect™ PI3K-mTOR Assay Kit (Merck Millipore Cat. No. FCCS025210) uses directly conjugated antibodies against phospho-Akt1/PKB (Ser473) and phospho-ribosomal protein S6 (Ser235) to analyze both upstream and downstream portions of this signaling cascade.

Bead-based assays, such as those using Luminex xMAP technology, have enabled the measurement of phosphorylation levels of up to 12 proteins simultaneously. Merck Millipore's MILLIPLEX MAP Akt/mTOR Panel (Merck Millipore Cat. No. 48-611) is a bead-based immunoassay that simultaneously detects 11 phosphoproteins (Figure 1) in the Akt/mTOR pathway in a single cell lysate sample, enabling the measurement of phosphorylation changes in this important pathway.

Here, we demonstrate the utility of the MILLIPLEX MAP Akt/mTOR 11-plex Panel in the analysis of Akt/mTOR signaling in cancer cell lines (HepG2, HEK293, and MCF7). All analytes were detected with good specificity, sensitivity and precision. In addition, we demonstrate successful detection of these phosphoproteins in human as well as mouse tissue samples (with the exception of phospho-IGF1R in mouse samples). Finally, our inhibitor studies show the utility of this panel in drug discovery research.

Methods

Tissue Culture. HepG2, HEK293, and MCF7 cells were cultured according to ATCC® guidelines in recommended media. Cells were grown to approximately 85% confluence; then serum starved for 4 hours prior to treatment with stimuli or compounds.

Sample Preparation. Cells were lysed and samples collected according to the MILLIPLEX MAP Cell Signaling Buffer and Detection Kit (Merck Millipore Cat. No. 48-602) instructions.

Microspheres. We developed the MILLIPLEX MAP Akt/mTOR 11-plex Panel by conjugating specific capture antibodies to microsphere beads purchased from Luminex Corporation. Each set of beads is distinguished by different ratios of two internal dyes, yielding a unique fluorescent signature to each bead set.

Human and Mouse Tissue Homogenization. Frozen tissue samples were weighed and placed on ice. Samples were homogenized with 1 mL lysis buffer per 50-100 mg mouse tissue or 1 mL lysis buffer per 30-50 mg of human tissue. Tissues were homogenized using the Omni International General Laboratory Homogenizer with OmniTip™- Plastic Generator Probes at setting level 2 for 30 seconds. Samples were then incubated with gentle rocking at 4° C for 15 minutes and centrifuged (10,000 g for 10 minutes at 4° C) to separate connective tissue, fat, ECM, etc. Supernatants were placed in new tubes. Protein concentration in each sample was determined by bicinchoninic acid (BCA) assay of sample aliquots—we observed typical protein recoveries of 5-10% of initial tissue mass. After diluting samples to 2 mg/mL in lysis buffer, they were transferred to 96-well plates in preparation for assay with the MILLIPLEX MAP kit.

Immunoassay Protocol. The multiplex assay was performed in a 96-well plate according to product instructions supplied for the MILLIPLEX MAP Akt/mTOR 11-plex Panel (Merck Millipore Cat. No. 48-611). The plate was first rinsed with 100 µL assay buffer. 25 µL of controls and samples and 25 µL beads were added to each well. Plates were incubated overnight at 4° C (alternatively can be incubated 2 hours at room temperature (RT)). Beads were washed twice with assay buffer, then incubated 1 hour at RT with biotinylated detection antibody cocktail. The detection antibody cocktail was replaced with 25 µL streptavidin-phycoerythrin (SAPE) and incubated for 15 minutes at RT. 25 µL of amplification buffer was added and incubated another 15 minutes at RT. Finally, the SAPE/amplification buffer was removed and beads were resuspended in 150 µL assay buffer. The assay plate was read and analyzed in a Luminex 200™ system. This is a compact unit consisting of an analyzer, a computer, and software (Luminex Corporation, Austin, TX).

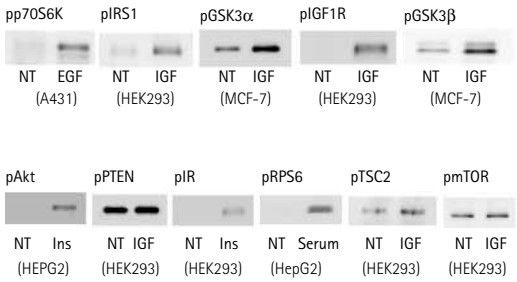
Results and Discussion

The MILLIPLEX MAP Akt/mTOR 11-plex Panel enabled the detection of phosphorylation events for all panel analytes with good specificity, sensitivity and precision (Figure 2). The assay provided high specificity, indicated by the detection of proteins at the expected molecular weights as shown by immunoprecipitation/Western blot (Figure 2A). Specificity was also demonstrated by detection of the correct isoforms of GSK3α/β and IR/IGF1R (Figure 2B). In addition, demonstrations of high signal-to-noise ratios (data not shown), sample linearity (Figure 2C) and precision (Figure 2D) lent support to the robustness of this kit.

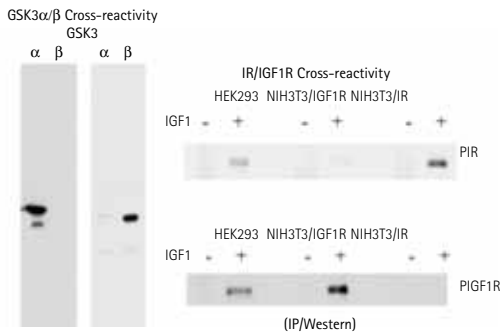
Figure 2.

Specificity, sensitivity, and precision of the MILLIPLEX MAP Akt/mTOR 11-plex Panel. Phosphorylated proteins were simultaneously detected in different cell lines treated with either insulin or IGF1. (A) Immunoprecipitation (IP) of phosphoproteins were performed with capture beads and detected by Western blotting with the biotinylated detection antibodies. (B) Isoform cross-reactivity tests were performed using human recombinant GSK3α/β by Western blotting or IR/IGF1R by IP/Western blotting. (C) Lysate titrations were performed on HepG2 cells treated with 10 µg/mL insulin for 15 minutes or MCF7 cells treated with 50 ng/mL IGF1 for 15 minutes. (D) Intra- and inter-assay coefficients of variation (CVs) were calculated and reported as percentages.

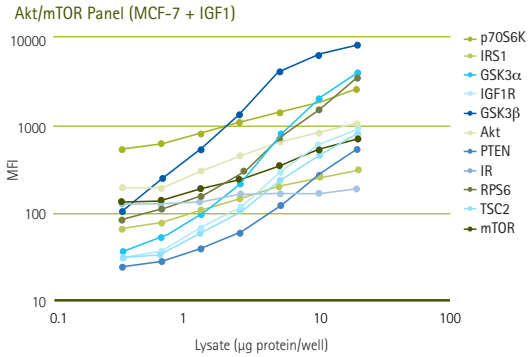
A. IP/Westerns

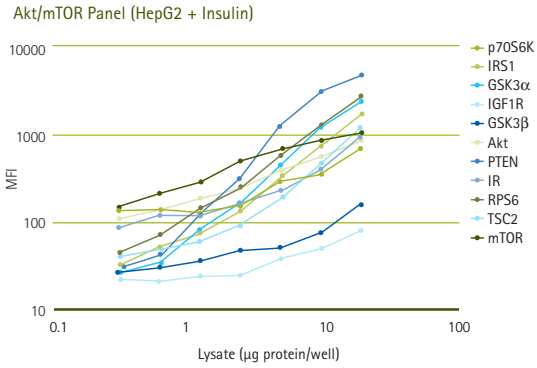


B. Cross-reactivity



C. Lysate Titrations





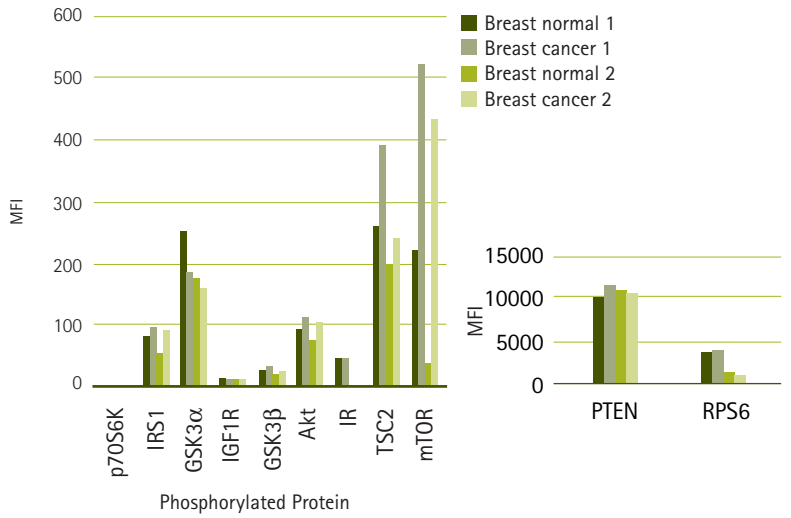
D. Precision

	Intra CV (%) n=16	Inter CV (%) n=16
p70S6K (Thr424)	5.7	16.8
IRS1 (Ser312)	7.4	9.6
GSK3α (Ser21)	5.8	9.8
IGF1R (Tyr1135/Tyr1136)	9.6	14.8
GSK3β (Ser 9)	10.4	10.8
Akt (Ser473)	5.8	9.7
PTEN (Ser380)	4.6	10.4
IR (Tyr1162/Tyr1163)	5.3	13.1
RPS6 (Ser235/Ser236)	6.4	14.0
TSC2 (Ser939)	6.9	10.6
mTOR (Ser2448)	8.1	14.1

All analytes in the MILLIPLEX MAP Akt/mTOR 11-plex Panel were detected in human and mouse tissues using the kit, with the exception of phospho-IGF1R, which is human-specific. Of interest is the observation that both breast cancer patients exhibited greater than a 2-fold increase in phosphorylation of mTOR compared to breast tissue from healthy subjects (Figure 3). This observation is consistent with cancer cells exhibiting a higher level of protein synthesis than normal cells. However, Akt, which is upstream of mTOR, and p70S6K and RPS6, both downstream of mTOR, did not exhibit a significant change in phosphorylation levels.

Figure 3.
Phosphorylated Akt/mTOR proteins were simultaneously detected in human (A) and mouse (B) tissue samples. (20 µg/mL). Total Akt/mTOR proteins were detected simultaneously in mouse (C) tissue samples using the MILLIPLEX MAP Total Akt/mTOR 11-plex Panel (available Q4 2011). Human matched breast normal and cancer tissue samples were purchased from Asterand. Mouse tissues from C57BL/6J males were purchased from Jackson Laboratory. Values were background-subtracted and reported as mean fluorescence intensity (MFI). "ND" represents "not detectable."

A. Phosphorylated Akt/mTOR proteins in human breast tissue samples



B. Phosphorylated Akt/mTOR proteins in mouse tissue samples

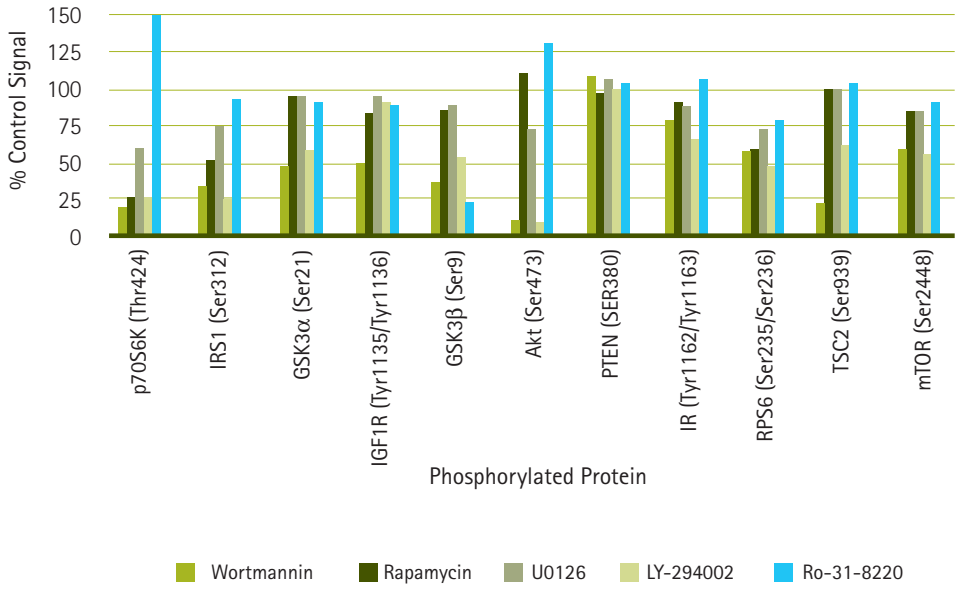
Mouse Tissues	p70S6K	IRS1	GSK3α	IGF1R	GSK3β	Akt	PTEN	IR	RPS6	TSC2	mTOR
Colon	ND	27	23	ND	42	ND	11094	53	7	4	ND
Pancreas	305	116	198	ND	62	215	10050	136	676	212	81
Pituitary	28	171	112	ND	176	198	10956	60	967	540	1183
Prostate	143	326	35	ND	176	163	10906	82	345	454	1102
Skeletal Muscle	186	665	38	ND	552	15	6643	12	265	374	636
Testicles	250	108	101	ND	57	52	13303	122	133	1346	1513
Liver	80	130	565	ND	51	226	13630	101	5696	562	458

C. Total Akt/mTOR proteins in mouse tissue samples

Mouse Tissues	p70S6K	IRS1	GSK3α	IGF1R	GSK3β	Akt	PTEN	IR	RPS6	TSC2	mTOR
Colon	157	61	25	ND	59	84	51	163	39	86	50
Pancreas	188	87	35	ND	37	140	51	220	50	82	61
Pituitary	2173	5434	2722	ND	269	2212	1752	509	241	370	305
Prostate	1556	5382	2673	ND	233	2309	1771	475	214	367	317
Skeletal Muscle	1324	4181	2704	ND	749	3475	2035	528	92	789	228
Testicles	1222	4241	2232	ND	619	3091	1821	426	85	608	171
Liver	1146	5387	2421	ND	160	2145	794	525	54	240	246

The addition of wortmannin, an inhibitor of PI3K, resulted in decreased levels of phosphorylation of several downstream targets such as Akt, mTOR, p70S6K and RPS6 (Figure 4). Rapamycin, an mTOR inhibitor, also inhibited its downstream targets p70S6K and RPS6. Consequently, these studies demonstrate that tumor development is complex and involves more than the dysregulation of a single pathway. This conclusion further underscores the importance of simultaneous measurement of multiple phosphoprotein targets and demonstrates the utility of the MILLIPLEX MAP Akt/mTOR 11-plex Panel.

Figure 4.
Phosphorylated Akt/mTOR pathway proteins were detected simultaneously in HepG2 cells treated with various inhibitors. Cells were pre-treated with 0.1 µM wortmannin, 0.1 µM rapamycin, 10 µM U0126 (MEK1/2 inhibitor), 50 µM LY-294002 (PI3K inhibitor), or Ro-31-8220 (PKC and GSK3β inhibitor) for 30 minutes prior to the addition of 10 µg/mL insulin for 15 minutes. Values were background-subtracted and reported as percent of vehicle and insulin-treated values, respectively.



Features of Merck Millipore's MILLIPLEX MAP Cell Signaling Assays

As shown by our Akt/mTOR signaling data, the complexity and number of protein targets involved in signaling events, as well as cellular responses, require multiplexed analysis of samples to achieve a complete, accurate picture of a signaling network. Merck Millipore's MILLIPLEX MAP Cell Signaling Assays enable the analysis of a greater number of intracellular analytes per well, saving valuable time and resources. Flexible assay formats include preconfigured multiplex panels as well as single-plex MAPmates which can be mixed and matched to meet individual needs.

MILLIPLEX MAP Cell Signaling Assay Kits

Traditional Cell Signaling Assay Features:

- Simultaneous measurement of multiple analytes in a single well
- Flexible configurations of multiplexing analytes to meet specific needs
- Options for both preconfigured multiplex panels and single-plex MAPmate kits
- Largest selection of intracellular analytes for detection with the Luminex system
- Kits or MAPmates for detecting phosphorylated and total protein
- GAPDH MAPmate can be purchased separately and plexed with other analytes for protein normalization
- All kits include lyophilized positive and negative control lysates

EpiQuant™ Cell Signaling Assay Features:

- Simultaneous measurement of multiple phosphorylation sites with picomolar sensitivities
- Absolute quantitation of multiple phosphorylation sites on the same protein simultaneously
- Absolute quantitation of both total and phosphoproteins simultaneously
- Enable customers to select only the analytes of interest from large panels
- Enable customers to design small- or large-plexed panels easily—using one catalogue number

References

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Related Products Available from www.millipore.com

Product Name	Catalogue Number
FlowCellect™ PI3K-mTOR Signaling Cascade kit	FCCS025210
FlowCellect P13K Activation Dual Detection Kit	FCCS025105

Products Available from www.merck4biosciences.com

Product Name	Catalogue Number
Wortmannin	681675
LY-294002	440202
Ro-31-8220	557520
Rapamycin	553210
U0126	662005

Featured Products Available from www.millipore.com

Product Name	Catalogue Number
MILLIPLEX MAP 11-plex Akt/mTOR Panel	48-611
MILLIPLEX MAP Cell Signaling Buffer and Detection Kit	48-602
IR (Tyr1162/Tyr1163) MAPmate	46-688
Akt (Ser473) MAPmate	46-677
PTEN (Ser380) MAPmate	46-679
mTOR (Ser2448) MAPmate	46-686
GSK3β (Ser9) MAPmate	46-690
p70S6K (Thr424) MAPmate	46-629
Total IR MAPmate	46-687
Total Akt MAPmate	46-675
Total PTEN MAPmate	46-678
Total mTOR MAPmate	46-685
Total GSK3β MAPmate	46-689
Total p70S6K MAPmate	46-630

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