

# Bird's-eye view of intracellular signal transduction pathways using customizable MILLIPLEX® MAP Cell Signaling Phospho/Total 2-Plex assays

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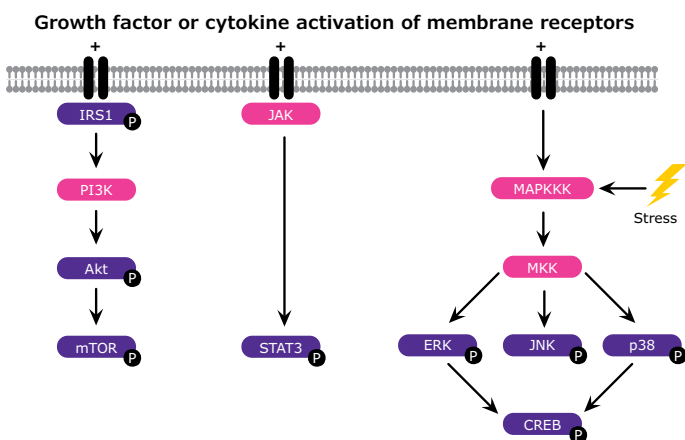
Dr. Saporita has over 10 years of experience in protein biochemistry and assay development. He earned his Ph.D. from Northwestern University and received postdoctoral training at Washington University in Saint Louis. He has several peer-reviewed publications in the areas of cancer biology and cell signaling. As part of the Research & Development team at Merck, Dr. Saporita designs new multiplex immunoassay panels for protein biomarker detection.

## Abstract

A wide range of phosphorylation events are critical for cellular processes, including growth and differentiation. Cells must be able to respond to extracellular and intracellular cues to modulate activation or repression of specific proteins. The Luminex® xMAP® platform enables multiplex analysis of both circulating and intracellular proteins, including phosphoproteins. Luminex® xMAP® technology is a bead-based assay that enables the simultaneous measurement of concentrations of multiple proteins in a single sample using Luminex® instrumentation to acquire and analyze resulting data.

MILLIPLEX® MAP Cell Signaling Phospho/Total 2-Plex assays enable the simultaneous detection of both phosphorylated and total protein in the same well without competition or interference. Assays for phosphorylated and total Akt1, Akt2, Akt3, (pan) Akt, CREB, ERK/MAPK 1/2, IRS1, JNK, mTOR, p38 and STAT3 are currently available (Table 1). Furthermore, MILLIPLEX® MAP Cell Signaling Phospho/Total 2-Plex assays can be combined, or “plexed” together, in a customizable panel, permitting the simultaneous study of multiple phosphorylated and total signaling proteins in the same well (Figure 1).

In this study, we use these 2-plex assays to monitor the responses of several signal transduction pathways to 3 compounds: a growth stimulus (IGF-1), a kinase inhibitor (LY294002), and a differentiation agent (retinoic acid). Our findings indicate these assays can sensitively detect even minor changes in protein phosphorylation and expression to determine which signaling proteins are impacted by a given treatment. These kits are a valuable tool to quickly generate a robust, fit-for-purpose multiplex assay. The kit protocols make it simple to mix-and-match 2-plex kits, creating a self-customized assay.



**Figure 1. Overview of MILLIPLEX® MAP Cell Signaling Phospho/Total 2-Plex Assays.**

Our 2-plex assays monitor activation of intracellular pathways in response to a variety of stimuli. This streamlined model illustrates some of the core components of the PI3K/Akt, JAK/STAT and MAPK pathways. Proteins included in the 2-Plex assay portfolio are shaded blue. Upstream signaling intermediates are shaded pink. The MAPKKK and MKK proteins responsible for activation of each of the MAPK pathways (ERK, JNK, and p38) have been grouped together using generalized terminology to simplify the illustration.

## Materials and Methods

### Cell Culture

All cell lines used in this study were obtained from ATCC and cultured according to the supplier's recommended protocols. For serum-starvation experiments, cells were cultured in serum-free media for 3 hours prior to the addition of the drug. Cells were lysed and samples collected according to the MILLIPLEX® MAP Cell Signaling Buffer and Detection Kit (Cat. No. 48-602MAG) instructions.

### Immunoassay

MILLIPLEX® MAP Phospho/Total 2-Plex assays were conducted in 96-well plates according to protocol. Briefly, plates were washed with assay buffer prior to addition of 25 µL of sample (20 µg cell lysate unless otherwise indicated) and 25 µL of 1X magnetic capture beads. Assays were incubated overnight at 4°C. The following day, plates were washed twice with 100 µL assay buffer, then incubated in 25 µL 1X biotinylated detection antibody cocktail for 1 hour. Detection antibody was removed, and streptavidin-phycoerythrin (SAPE) was added for 15 minutes. Cell Signaling Amplification buffer was added to SAPE, and plates were incubated for an additional 15 minutes. All incubation steps were carried out on a plate shaker at medium speed. Assay plates were read and analyzed on a Luminex® 200™ system, and mean fluorescence intensity (MFI) data was collected.

The names and catalog numbers for the 2-plex assays used in this report are provided in Table 1. Individual (un-pooled) capture beads and detection antibodies were used to compare "single-plex" and 2-plex MFI measurements of total and phosphoprotein.

Cell Signaling Phospho/Total 2-plex kits can be combined to assemble a multitude of panels with different plex sizes, enabling researchers to customize an assay to meet their specific needs. Assay protocols are easily amenable to plexing. All magnetic beads and biotin-labeled detection antibodies are provided as 20X stocks. For each 2-plex kit to be added to the assay, beads and detection antibodies need to be diluted to 1X in the appropriate volume of assay buffer. When running a single 2-plex assay, 150 µL of beads/detection antibody is added to 2.85 mL of assay buffer. If three phospho/total 2-plex kits are combined, then 150 µL of each bead/detection antibody would be added to 2.55 mL of assay buffer. Up to 8 different phospho/total 2-plex kits can be combined to generate a 16-plex assay. Alternatively, Cell Signaling MAPmates™ can be plexed with the 2-plex assays. In particular, β-tubulin (46-713MAG) and GAPDH (46-667MAG) MAPmates™ can be used as "loading controls" to normalize MFI data.

Each phospho/total 2-plex assay is compatible with other phospho/total 2-plexes, with the exception of the Akt isoform assays. Three highly homologous Akt isoforms exist, with different physiological roles and patterns of

expression<sup>1</sup>. Each Akt isoform phospho/total 2-plex assay detects the intended isoform with high specificity (data not shown). These assays can be successfully plexed with all other (non-Akt) 2-plex kits, but not with one another. As such, we independently tested the customizable 16-plex assay (8 phospho/total 2-plex assays) with each of the Akt isoform and pan Akt kits.

## Results and Conclusions

Proteins can be regulated at the level of total protein expression and by phosphorylation events that may modulate protein activity. The ability to measure both total protein and phosphoprotein within the same sample in an immunoassay is advantageous. Further, being able to identify the relative proportion of protein in the phosphorylated state enables researchers to normalize their data. In an immunoassay format, however, it is crucial that the antibody pairs for the total protein and phosphorylated epitope do not compete or interfere with one another. To demonstrate the feasibility of this approach, we compared MFI measurements for phosphoproteins and total proteins run either individually or in a 2-plex format, using the unstimulated and stimulated cell lysates associated with each particular kit as controls.

Total protein and phosphoprotein MFIs were nearly identical when tested in either a "single-plex" or 2-plex assay (Figure 2A and 2B). This pattern was consistently observed, whether assessing unstimulated or stimulated cell lysates, demonstrating that both the single-plex and 2-plex assays had similar sensitivities. When MFI values were compared for individual analytes, the data showed a striking correlation ( $R^2=0.9949$ ) regardless of signal intensity (Figure 2C).

This data demonstrates that both the total protein and its phosphorylated epitope can be detected simultaneously and independently in the same sample, without competition or interference. In general, the total protein MFI was typically greater than the phosphoprotein MFI, which would be predicted, as only a portion of the total protein will be phosphorylated. However, there are instances when the phosphoprotein MFI is greater than that of the total protein, as was observed with JNK. It is important to note that other factors besides protein abundance may affect MFI measurement, such as the presence of multiple isoforms or antibody pair avidity for a target.

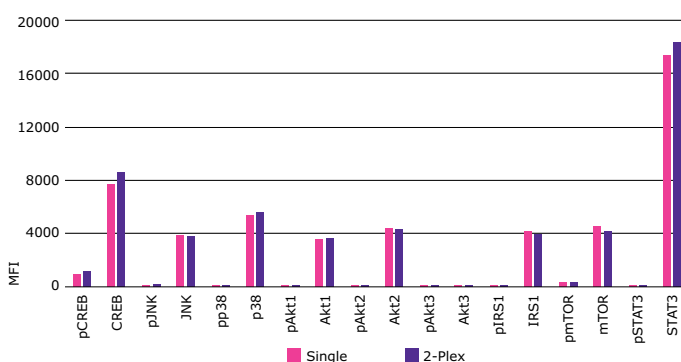
Next, we determined whether the individual 2-plex kits could be pooled to create a "customizable" multiplex assay capable of measuring multiple phosphoproteins and total proteins from several intracellular signal transduction pathways simultaneously. HeLa:TNFα+CaIa (Cat. No. 47-230) and HepG2:Insulin (Cat. No. 47-227) were used as stimulated cell lysates to compare the 2-plex assay results with those of a customized 16-plex assay. As expected, there were some differences in the MFI values observed for specific analytes when comparing the 2-plex and 16-plex assays.

Panel Name	Phosphorylation Site	Cat. No.
Akt1 Phospho/Total – 2 Plex	Ser473	48-631MAG
Akt2 Phospho/Total – 2 Plex	Ser474	48-632MAG
Akt3 Phospho/Total – 2 Plex	Ser472	48-633MAG
Akt Phospho/Total – 2 Plex	Ser473/Ser474/Ser472	48-618MAG
CREB Phospho/Total - 2 Plex	Ser133	48-628MAG
Erk/MAPK 1/2 Phospho/Total - 2 Plex	Thr185/Tyr187	48-619MAG
IRS1 Phospho/Total - 2 Plex	Ser636	48-626MAG
JNK Phospho/Total - 2 Plex	Thr183/Tyr185	48-622MAG
mTOR Phospho/Total - 2 Plex	Ser2448	48-625MAG
p38 Phospho/Total - 2 Plex	Thr180/Tyr182	48-624MAG
STAT3 Phospho/Total - 2 Plex	Tyr705	48-623MAG

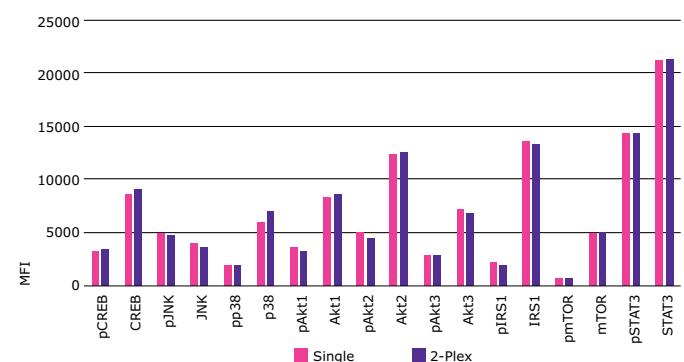
**Table 1.**

MILLIPLEX® MAP Phospho/Total 2-Plex assays used.

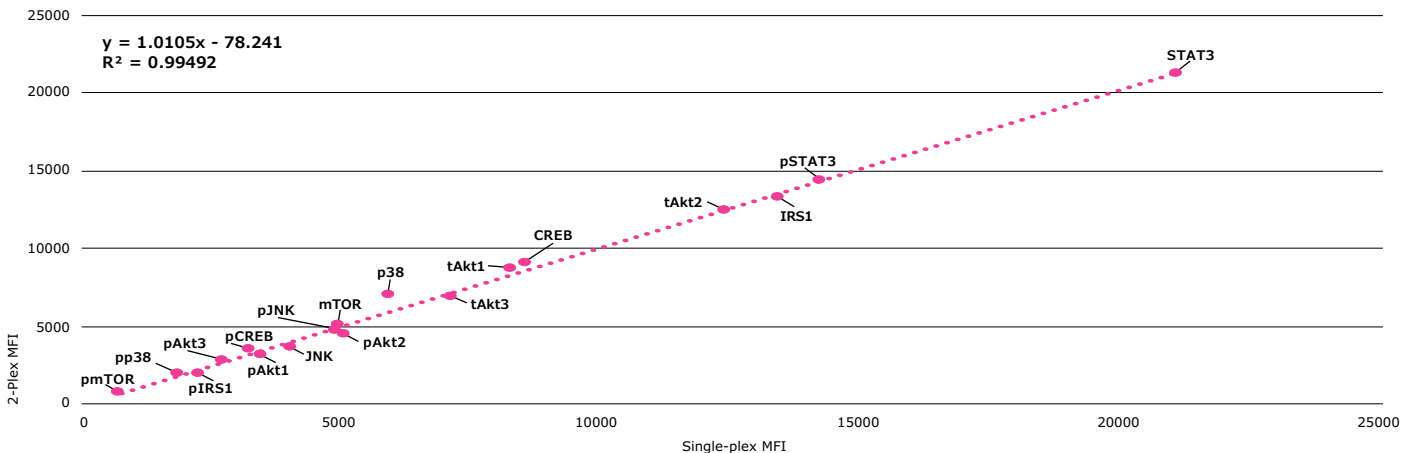
## 2A. Unstimulated Cell Lysates



## 2B. Stimulated Cell Lysate Controls



## 2C.



**Figure 2.**

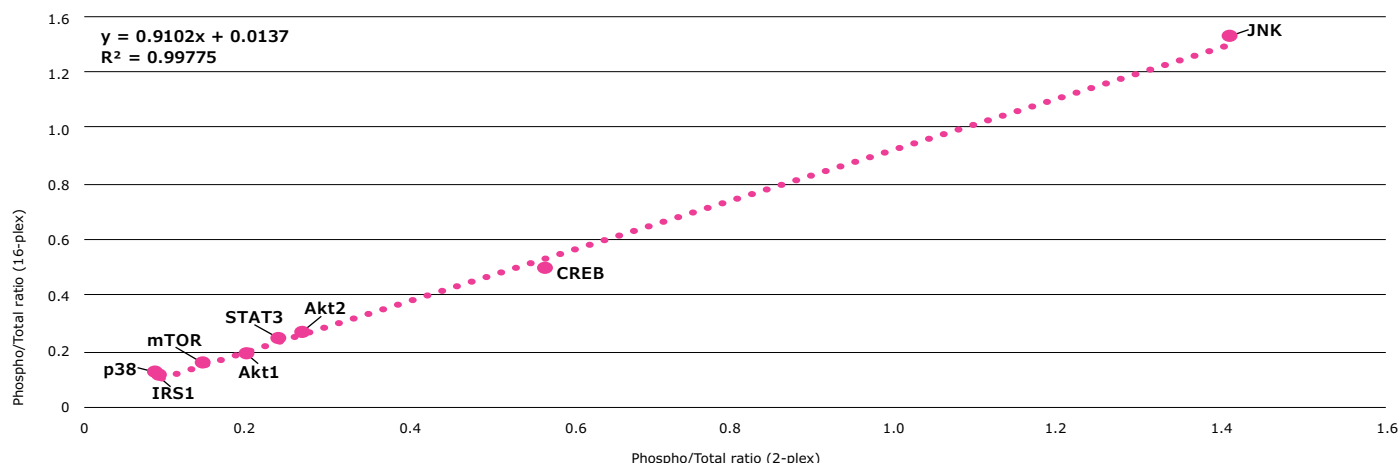
Plex-ability of phospho and total antibody pairs. (2A) MFI values in unstimulated cell lysates. HeLa unstimulated cell lysates (Cat. No. 47-205) were used for the measurement of Phospho/Total CREB, JNK, p38, Akt1, Akt2, Akt3, IRS1 and STAT3. HepG2 unstimulated cell lysate (Cat. No. 47-239) was used for the measurement of Phospho/Total mTOR. (2B) MFI values in stimulated cell lysates. HeLa: IFN $\alpha$  (Cat. No. 47-226) was used for the measurement of Phospho/Total STAT3. HeLa: TNF $\alpha$ +CaIa (Cat. No. 47-230) was used for the measurement of Phospho/Total CREB. HeLa: HS/Ars (Cat. No. 47-211) was used for the measurement of Phospho/Total p38 and JNK. HepG2:Insulin (Cat. No. 47-227) was used for the measurement of Phospho/Total mTOR. MCF7:IGF-1 (Cat. No. 47-216) was used for the measurement of Phospho/Total IRS1. HEK293:Serum (Cat. No. 47-233) was used for the measurement of Phospho/Total Akt1, Akt2, and Akt3. (2C) Correlation of single-plex vs. 2-plex MFI values in stimulated cell lysates. The data from Figure 2B was plotted to compare the MFI values obtained by 2-plex assays with those obtained when a “single-plex” assay was run for each total or phosphoprotein.

Analyzing the ratio of phosphoprotein to total protein demonstrated a strong, linear relationship between the measurement of most phospho/total analytes when transitioning from a 2-plex assay to a customized 16-plex assay ( $R^2 = 0.9977$ , Figure 3A). Two phospho/total 2-plex assays (ERK and Akt3) experienced larger variations in MFI when transitioning to the customized 16-plex assay. Phospho-ERK experienced a reduction in MFI, with minimal change in total ERK, whereas both phospho-Akt3 and total Akt3 exhibited decreases in MFI signal in the customizable 16-plex assay format (data not shown). While these reductions in signal intensity may have disrupted the ratio of phosphoprotein to total protein, the 16-plex panel format did not interfere with the relative detection of these proteins when comparing cell lysates with varying degrees of pathway activation (Figure 3B, 3C). For example, HEK293:Serum lysate (Cat. No. 47-233) has a higher ratio of phospho-ERK/total ERK compared to A549:Camptothecin lysate

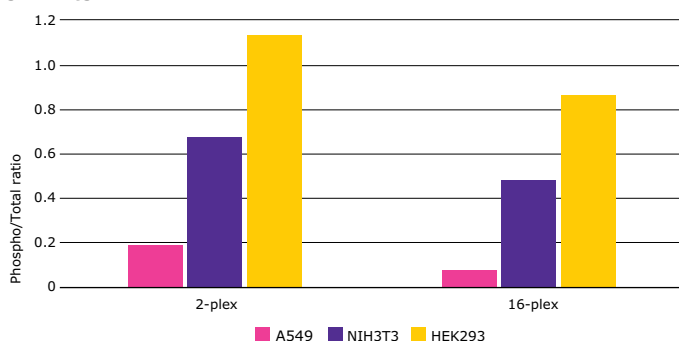
(Cat. No. 47-218), regardless of whether a 2-plex assay or 16-plex assay was used. Thus, the general pattern of protein phosphorylation for ERK and Akt3 is preserved when integrating these assays into a customizable 16-plex panel.

Commercially available MILLIPLEX® MAP Cell Signaling lysates were then screened using the customizable 16-plex assay. This generated a profile in which the relative expression and phosphorylation of a signaling protein can be compared across a breadth of cell lines and treatments (Figure 4). While the majority of tested lysates originated from human cell lines, the inclusion of NIH3T3:Anisomycin (Cat. No. 47-219) and rat heart lysates demonstrate the 2-plex assays can also effectively detect mouse and rat proteins. Analysis of the cell lysate profile illustrated both the variation in protein expression between cell lines, and the differences in protein phosphorylation patterns in response to diverse stimuli.

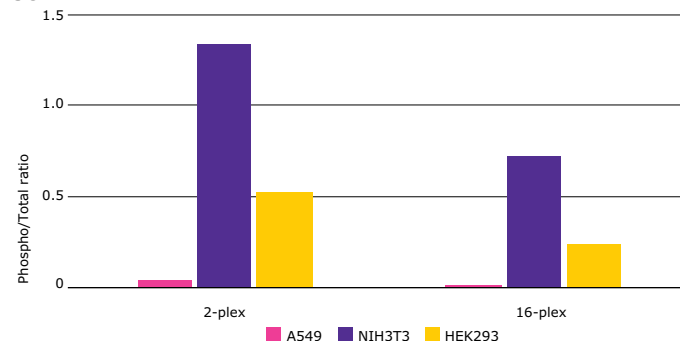
### 3A.



### 3B. Akt3



### 3C. ERK



**Figure 3.**

Plex-ability of combined 2-plex assays. (3A) Correlation of 2-plex versus 16-plex measurements in stimulated cell lysates. HepG2:Insulin was used as a stimulated cell lysate for Phospho/Total JNK, mTOR, STAT3, Akt1, and Akt2. HeLa:TNF $\alpha$ +CalA was used as a stimulated cell lysate for Phospho/Total CREB, p38 and IRS1. The phospho/total (P/T) ratio was calculated by dividing the MFI of the phosphoprotein over the MFI of the total protein. The reported 16-plex values include MFI data averaged from separate 16-plex assays containing either Akt1 (Cat. No. 48-631MAG), Akt2 (Cat. No. 48-632MAG) or (pan) Akt (Cat. No. 48-618MAG) beads and detection antibody. (3B) and (3C) Phospho/Total Akt3 and Phospho/Total ERK assays are affected by multiplexing. The Phospho/Total ERK and Phospho/Total Akt3 assays displayed MFI values that experienced fluctuation when moving from 2-plex to 16-plex. The phospho/total protein ratios for ERK and Akt3 were analyzed in three cell lysates: A549:Camptothecin (Cat. No. 47-218), NIH3T3:Anisomycin (Cat. No. 47-219) and HEK293:Serum (Cat. No. 47-233). While the absolute phospho/total ratio for both proteins changed, the overall phosphorylation pattern for each analyte was preserved.



**Figure 4.**

Evaluating MILLIPLEX® MAP Cell Signaling lysates with a customizable 16-plex panel comprised of Phospho/Total 2-plex assays. Cell lysates were screened by 16-plex assay, and the total and phosphoprotein MFI measurements for each of the 2-plex assays were collected and illustrated in separate graphs.

To demonstrate the sensitivity of the customizable 16-plex panel, we examined changes in phosphorylation in a dose-response assay. MCF-7 human breast cancer cells were serum-starved for 3 hours, then treated with the indicated doses of IGF-1 for 15 minutes. Cell lysates were collected, and the relative levels of phosphorylated proteins and total proteins were measured in a 16-plex panel comprising pooled reagents from multiple 2-plex assays. Not all proteins responded to the short IGF-1 stimulus; however, ERK, Akt and JNK exhibited induction of phosphorylation. In addition, phosphorylation of these proteins was sensitive to increasing concentrations of IGF-1 (Figure 5).

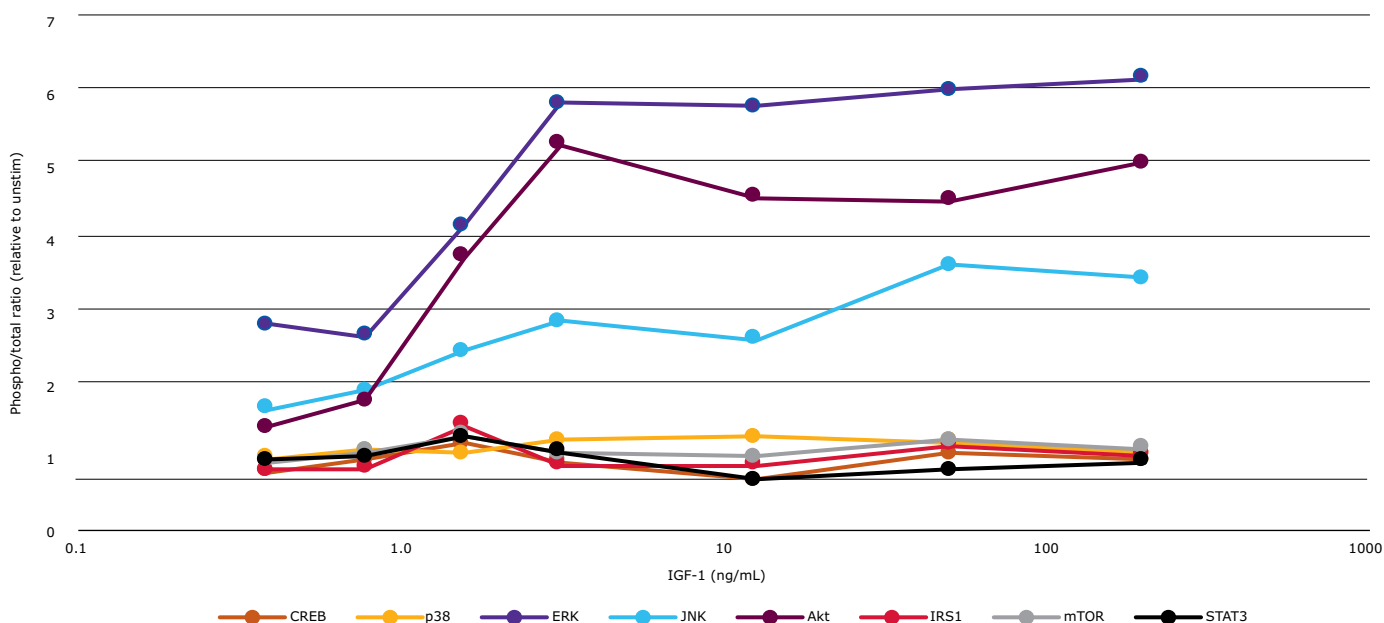
The effect of the PI3K inhibitor LY294002 on IGF-1-induced phosphorylation was then examined. MCF-7 cells were serum-starved and pre-treated with LY294002 for 20 minutes prior to the addition of IGF-1. After a 15-minute IGF-1 stimulation, cells were lysed and analyzed by a 16-plex assay with either Akt1 or Akt2. In MCF-7 cells, Akt3 expression was undetectable (Figure 6A), consistent with published reports<sup>2</sup>. Consequently, the phospho/total Akt3 2-plex assay was not included in the 16-plex analysis. Akt1, Akt2, ERK and JNK exhibited >2-fold inductions of phosphorylation in response to IGF-1. Pre-treatment with 20  $\mu$ M LY294002 completely abrogated the IGF-induced phosphorylation of Akt1 and, to a lesser extent, attenuated the activation of Akt2 (Figure 6B). This result suggests a differential sensitivity of the Akt isoforms to PI3K inhibition. In contrast to the Akt isoforms, phospho-JNK and phospho-ERK were

further stimulated by LY294002, suggesting a potential compensatory feedback mechanism in the cross-talk between these intracellular signaling pathways<sup>3</sup>.

Selective activation of signal transduction pathways also plays an integral role in differentiation. SH-SY5Y cells were used as a model to study intracellular signaling in the context of neuronal differentiation. We were particularly interested in which Akt isoforms would be activated. Briefly, SH-SY5Y cells were treated with retinoic acid (RA) for 3 days to induce neuronal differentiation before cells were collected, lysed and evaluated by multiplex assay. RA-induced differentiation of SH-SY5Y cells was accompanied by phosphorylation of all 3 Akt isoforms, with Akt2 and Akt3 displaying the greatest induction (Figure 7). Multi-pathway analysis demonstrated co-induction of phospho-JNK in the RA-treated SH-SY5Y cells, consistent with the role of JNK in RA-mediated differentiation<sup>4</sup>. Phosphorylation of mTOR and STAT3 also increased in the RA-treated cells.

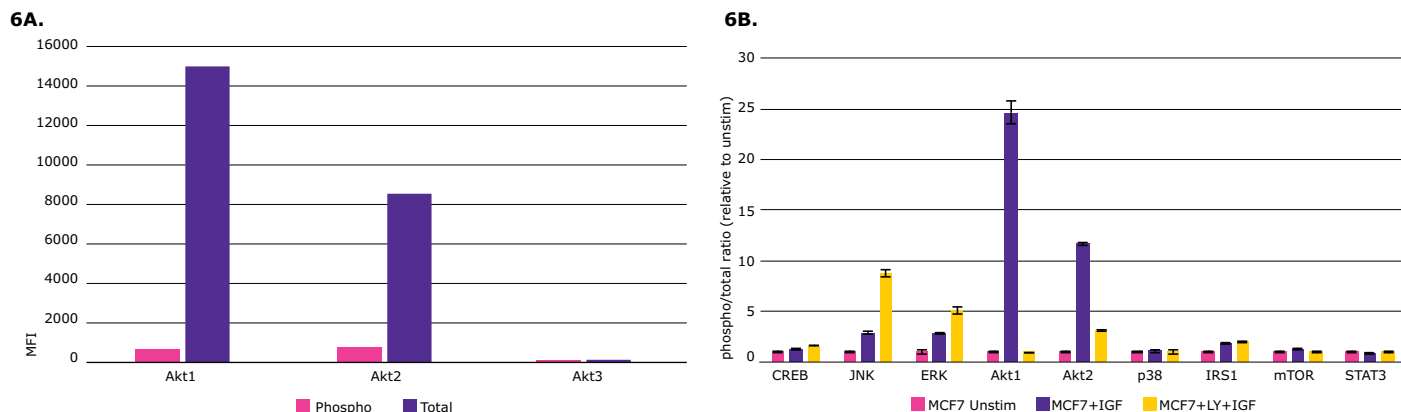
## Summary

The data presented here demonstrate the accuracy and utility of analyzing signal transduction pathways with the MILLIPLEX® MAP Cell Signaling Phospho/Total 2-Plex assays. These kits enable simultaneous measurement of total protein and phosphoprotein expression and activation. Further, the “plex-ability” of these 2-plex assays allows researchers to create customizable panels to get a broad overview of signaling events, or interrogate specific pathways of interest.



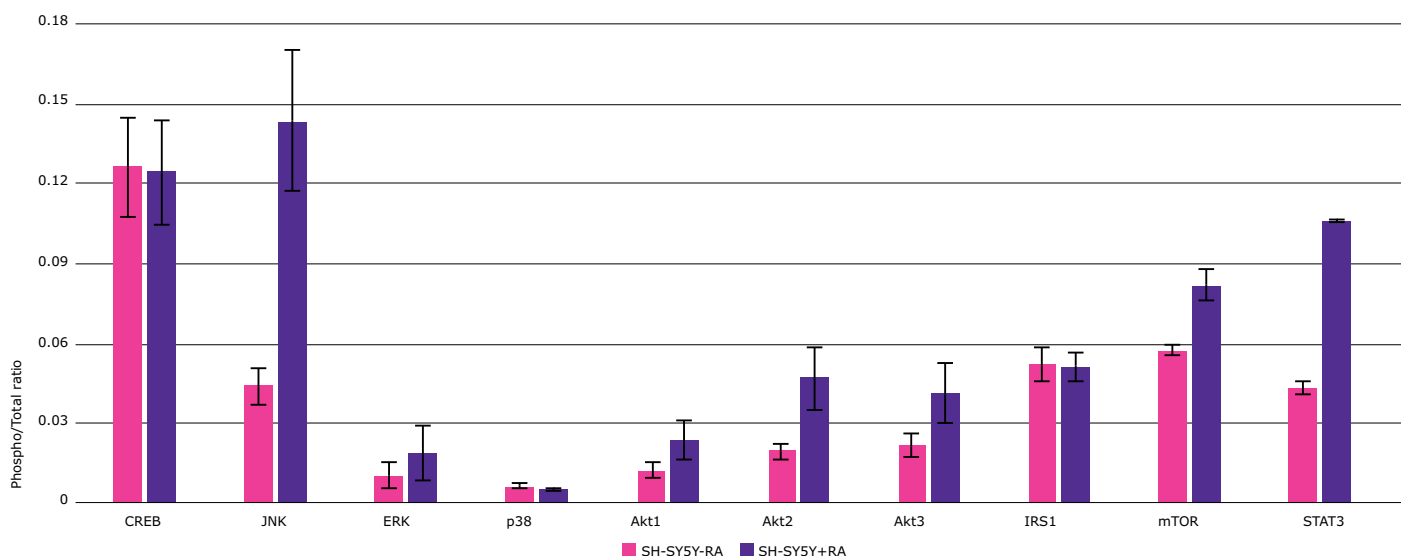
**Figure 5. IGF dose response in MCF7.**

MCF-7 human breast cancer cells were serum-starved for 3 hours prior to addition of IGF-1 at the indicated concentrations for 15 minutes. Cell lysates were collected according to MILLIPLEX® MAP assay protocols, and samples (20  $\mu$ g/well) were measured using a 16-plex assay composed of several phospho/total 2-plex assays. The ratio of phosphoprotein to total protein was calculated using median MFI values. These ratios were then normalized to the phospho/total protein ratios from unstimulated MCF-7 cells.



**Figure 6.**

LY294002 preferentially inhibits Akt1 phosphorylation in MCF7 cells. (6A) MCF-7 cells express Akt1 and Akt2 but not Akt3. Lysates were collected from unstimulated MCF7 cells. Phospho/Total 2-plex assays for Akt1, Akt2 and Akt3 were used to measure the relative expression of the Akt isoforms. (6B) LY294002 preferentially inhibits Akt1 phosphorylation in response to IGF-1 in MCF-7 cells. MCF7 cells were cultured in the presence or absence of 20  $\mu$ M LY294002 for 20 minutes before the addition of IGF-1. Lysates were collected and subjected to analysis using a customizable 16-plex panel composed of the indicated phospho/total 2-plex assays.



**Figure 7.**

SH-SY5Y differentiation is accompanied by activation of multiple signaling proteins. SH-SY5Y cells were treated with retinoic acid (RA) for 3 days to stimulate neuronal differentiation. After 3 days, cells cultured in the presence or absence of RA were collected and lysed according to MILLIPLEX® MAP assay protocols. MFI measurements were obtained from 16-plex panels using pooled 2-plex assays (Akt isoform 2-plex assays were run both individually and in separate 16-plex panels), and the ratio of phosphoprotein to total protein was calculated for each analyte.

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