

Validation and Application of Bio-Plex Pro[™] Cell Signaling Assays

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Introduction

Cell signaling mediated through the activation of protein kinases can induce changes in metabolism, transcription, cell cycle progression, cytoskeletal rearrangement and cell movement, apoptosis, and differentiation (Chang and Karin 2001). Protein phosphorylation also plays a critical role in intercellular communication during fetal development and in the functioning of the nervous and immune systems (Yates 2011, Boulanger et al. 2001). Analysis of the human genome has revealed the presence of 518 different protein kinases (Manning et al. 2002). Mutations in 218 of these kinases appear to be associated with human disease; therefore, understanding which cell signaling pathways are involved in particular diseases allows researchers to develop targeted therapeutics with improved efficacy and safety and enables clinicians to better identify patients who may benefit from the therapy. Indeed, protein kinases have emerged as an important group of biomarkers and drug targets making them a focus of in vitro biomarker assay development.

To advance the study of cell signal transduction, we developed Bio-Plex Pro cell signaling assays for the semiquantitative detection of phosphoproteins and total target proteins in cell and tissue lysates (Bio-Rad bulletin 6285). These assays utilize Luminex xMAP technology, employing a magnetic bead-based workflow to enable multiplex detection of proteins in a single well of a 96-well microplate. For example, using Bio-Rad Laboratories' Bio-Plex Pro panels, Alexopoulos et al. reported ~6,500 measurements from a 96-well plate assayed for 17 phosphoproteins and 50 cytokines (Alexopoulos et al. 2010). Here, we describe our assay validation and the successful application of these Bio-Plex panels in measuring drug effects on potential therapeutic targets in key cell signaling pathways.

Materials

The panel includes 23 assays for the quantitation of phosphorylated protein targets and 11 assays for total proteins (i.e., both the phosphorylated and unphosphorylated forms of the target protein; Table 1 and bulletin 6285). These markers were selected for their direct relevance to cell signaling. The assay configurations are modeled after the previously developed cytokine and diabetes assays (bulletins 6159 and 6249), with an all-in-one configuration for custom assays in a 96-well plate format.

Method

The principle of the Bio-Plex Pro bead-based assays is similar to capture sandwich immunoassays (Zhou and Geng 2011, Yeung et al. 2011). The capture antibody-coupled beads are first incubated with the sample followed by incubation with biotinylated detection antibodies. After washing away the unbound biotinylated antibodies, the beads are incubated with a reporter streptavidin-phycoerythrin conjugate (SA-PE). Washing is omitted for assays of 6-plex or less. The beads are then passed through the Bio-Plex[®] reader, which measures the fluorescence of the bound SA-PE on each bead. Data is expressed as the median fluorescence intensity (MFI) for a given bead population. All assay incubations are performed at room temperature as described in the instruction manual (Bio-Rad part number 10024929). In our study, all washes were performed using a Bio-Plex Pro wash station with cycles of 200 μ l of wash buffer per well. Analyses were performed using the Bio-Plex 200 system at the high PMT setting with data acquisition in Bio-Plex Manager[™] software.

Assay Performance Characteristics

The Bio-Plex Pro cell signaling assays were evaluated for specificity, sensitivity, dynamic range, intra- and inter-assay percentage coefficients of variation (%CV), working assay range, cross-reactivity, and species specificity (Table 1).

Table 1: Assay Performance

Phosphoprotein Assays	P/B Ratio*	Intra-assay %CV	Inter-assay %CV	Dynamic Range, µg/well**	Reactive Species***
Akt (Ser ⁴⁷³)	65	2.9	4.5	0.15–10	H, M, R
Akt (Thr ³⁰⁸)	34	3.3	6.1	0.15–10	H, M, R
c-Jun (Ser ⁶³)	295	5.8	8.4	0.15–10	H, M, R
EGFR (Tyr ¹⁰⁶⁸)	98	14.1	17.7	0.15–10	H
EGFR (Tyr ¹¹⁷³)	30	3.9	6.3	0.15–10	H
Erk1/2 (Thr ²⁰² /Tyr ²⁰⁴ , Thr ¹⁸⁵ /Tyr ¹⁸⁷)	48	4.3	4.9	0.15–10	H, M
GSK-3α/β (Ser ²¹ /Ser ⁹)	19	2.9	5.4	0.15–10	H, M, R
HER-2 (Tyr ¹²⁴⁸)	49	10.1	9.1	0.15–10	H, M, R
IGF-IR (Tyr ¹¹³¹)	68	4.8	6.5	0.15–10	H
IκB-α (Ser ³² /Ser ³⁶)	103	6.8	8.9	0.15–10	H
JNK (Thr ¹⁸³ /Tyr ¹⁸⁵)	73	4.7	4.1	0.15–10	H
MEK1 (Ser ²¹⁷ /Ser ²²¹)	44	1.9	5.5	0.15–10	H, M, R
NF-κB p65 (Ser ⁵³⁶)	20	10.7	13.5	0.30–10	H, M, R
p38 MAPK (Thr ¹⁸⁰ /Tyr ¹⁸²)	62	3.1	6.4	0.15–10	H, M, R
p70 S6 Kinase (Thr ⁴²¹ /Ser ⁴²⁴)	11	2.9	20.1	0.15–10	H, M, R
p70 S6 Kinase (Thr ³⁸⁹)	196	8.4	10.3	0.15–10	H, M, R
PDGFR-α (Tyr ⁷⁵⁴)	40	5.0	6.2	0.04–10	H, M
PDGFR-β (Tyr ⁷⁵¹)	142	8.1	10.3	0.04–10	H, M, R
Smad2 (Ser ⁴⁶⁵ /Ser ⁴⁶⁷)	14	4.4	8.8	0.30–10	H, M, R
Stat1 (Tyr ⁷⁰¹)	18	3.6	7.8	0.15–10	H
Stat3 (Tyr ⁷⁰⁵)	18	2.5	6.8	0.15–10	H, M, R
Stat3 (Ser ⁷²⁷)	89	7.0	10.1	0.15–10	H, M, R
VEGFR-2 (Tyr ¹¹⁷⁵)	614	8.3	11.7	0.15–10	H, M
Total Target Assays	P/B Ratio*	Intra-assay %CV	Inter-assay %CV	Dynamic Range, µg/well**	Reactive Species***
Akt	–	3.8	8.4	0.15–10	H, M, R
c-Jun	–	4.0	4.7	0.15–10	H
Erk1/2	–	3.4	9.2	0.15–10	H, M
GSK-3β	–	2.9	4.1	0.04–2.5	H, M, R
HER-2	–	5.6	4.2	0.04–10	H, M, R
IκB-α	–	1.9	3.1	0.15–10	H
JNK	–	1.9	4.5	0.30–10	H
MEK1	–	1.8	3.9	0.15–10	H, M, R
p38 MAPK	–	4.3	9.6	0.30–10	H, M, R
p70 S6 Kinase	–	2.0	3.6	0.15–10	H, M, R
Smad2	–	3.9	16.9	0.15–10	H

* Signal ratio (positive/background) of the stimulated cell lysate divided by the negative control cell lysate. Analyses performed at high PMT on a Bio-Plex 200 system.

** The range of total lysate protein in which the analyte can be detected.

*** H = Human; M = Mouse; R = Rat

Specificity, Cross-Reactivity, and Reactive Species

The majority of the Bio-Plex Pro cell signaling assays were developed and validated with antibodies from Cell Signaling Technology (www.cellsignal.com), a company well known for diverse and high-quality cell signaling antibodies. To confirm the specificity of the assays, we analyzed the performance of all detection antibodies using western blotting (WB) against positive and negative control lysates. In parallel, we analyzed the same lysates in the Bio-Plex suspension array system, and we selected only the assays that showed good correlation with the WB results for further development. We found that WB analysis was complicated by the presence of multiple bands in some cases. However, when the appropriate molecular weight (MW) band was quantified in each case, we found excellent specificity and correlation with the Bio-Plex assays.

The simultaneous presence of multiple detection assays in a given well may result in erroneous results due to nonspecific binding or cross-reactivity. These assays were extensively optimized to minimize nonspecific binding. However, homologies among proteins in the same family, especially in the phosphorylated domains, make the multiplexing of some assays impractical; for example, EGFR and Her-2, both in the ErbB family, share 83% homology in the tyrosine kinase domain. Detailed information on assay cross-reactivity levels is provided in Bulletin 6285.

Species cross-reactivity was predicted based on 100% sequence homology for the capture and detection antibodies. Reactivity was confirmed for applicable targets using the NIH 3T3 mouse and PC12 rat cell lines.

Sensitivity

We quantified detection sensitivity in two ways. First, we measured positive signal as the fold increase of a positive sample MFI (P) divided by the negative control MFI (B) at a protein concentration of 200 µg/ml to obtain the P/B ratio (Table 1). The positive samples were lysates prepared from cell lines with signal transduction pathways activated by the following stimuli: EGF, IGF, NGFβ, PDGF, VEGF, TNF-α, IFN-α, and UV irradiation. The negative control was HeLa cell lysate treated with an Mn²⁺-dependent protein phosphatase (Lambda PP, New England Biolabs). Second, we evaluated the detectability of serially diluted samples over a blank (detection antibody diluent) plus two standard deviations. The majority of the assays detected targets at levels as low as 0.15 µg/well, with some assays exhibiting detection at 0.04 µg/well.

To compare the sensitivity of the Bio-Plex Pro cell signaling assays with that of WB, we serially diluted the positive lysates and measured the signals obtained with both methods. Although we used an ultrasensitive chemiluminescence detection reagent for WB, we found that the Bio-Plex assay was at least ten times more sensitive (bulletin 6285).

Precision

To assess assay precision, we calculated intra- and inter-assay percentage coefficients of variation (%CV). Four operators performed twelve assays in 3- to 12-plex formats using four different Bio-Plex 200 readers. Each assay was run three times independently over three days against several positive and negative cell lysate controls at protein concentrations of 20 µg/ml and 200 µg/ml. Two sample types were tested: frozen and reconstituted lyophilized lysate. More than 2,000 replicates were analyzed, covering all positive and negative samples. In this vast number of analyses, 89% of both the intra-assay and inter-assay %CVs were less than 15% and 97% were less than 25%. Table 1 lists the intra-assay and inter-assay %CVs for positive lysates at 200 µg/ml.

Application: Elucidation of Cell Signaling Pathways and Effects of Small Molecule Kinase Inhibitors

To evaluate the performance and applicability of Bio-Plex Pro cell signaling assays, we conducted a study in the laboratory of Prof. Douglas Lauffenburger at the Massachusetts Institute of Technology. For all experiments, we seeded HepG2 cells in 24-well plates at a density of 1,500 cells/mm² and serum-starved them overnight. Two 12-plex panels were designed by Dr. David C. Clarke to measure the levels of 12 phosphoproteins and total proteins at a lysate concentration of 200 µg/ml using 50 µl of sample per well. The beads were read at high PMT with a Bio-Plex 200 reader and MFI data were obtained as duplicate means with standard deviations.

In the first study, we stimulated the cells for 30 min with a variety of cytokines including IL-1α (200 ng/ml), IL-1β (200 ng/ml), IL-6 (200 ng/ml), IGF-1 (100 ng/ml), TGF-α (200 ng/ml), TGF-β (2.5 ng/ml), IFN-γ (100 ng/ml), and TNF-α (300 ng/ml), both alone and in selected combinations for a total of 10 treatments. Changes in signaling pathway responses were measured with 12-plex total protein and 12-plex phosphoprotein assays. As expected, the numbers of phosphorylated proteins and their degree of phosphorylation varied according to the treatments. For example, treatment with TGF-β led to increased levels of phosphorylated Smad2 only, whereas treatment with IFN-γ resulted in the phosphorylation of Stat1 (data not shown). Conversely, treatment with IL-1α, IL-1β, TGF-α, or their combinations led to widespread changes in the phosphorylation levels of proteins including c-Jun, Erk1/2, IκB-α, JNK, p38 MAPK, p70 S6 Kinase (p70S6k), Akt, and GSK3α/β (data not shown). Thus, applying diverse cytokines to cells followed by multiplexed measurements of the downstream phosphoproteins allows for the investigation of many different pathways in a single Bio-Plex study. For simplicity, only a small subset of the total assay data is presented herein.

As part of the same study and to further demonstrate the specificity of the Bio-Plex Pro cell signaling assays, we stimulated HepG2 cells with IL-1α and IGF-1 in the presence of three small-molecule kinase inhibitors designed to block the phosphorylation of downstream targets (Figure 1). In all experiments, the HepG2 cells were preincubated for 30 min with the inhibitors followed by the addition of cytokines for an additional 30 min. First, the HepG2 cells were treated with IL-1α and 5Z-7-oxozeaenol (4 µM), a specific, irreversible inhibitor of TGF-β-activated kinase 1 (TAK1) (Ninomiya-Tsuji et al. 2003). Consistent with previous reports, we found that 5Z-7-oxozeaenol attenuated IL-1α activation of Erk1/2, p38 MAPK, JNK, c-Jun, and IκB-α, confirming upstream regulation of pathways involving these five kinases by TAK1 (Figure 2).

Second, we treated HepG2 cells with IL-1α and BMS-345541 (28 µM), a compound that binds and inhibits IκB kinase (IKK) (Burke et al. 2003). Because IKK phosphorylates IκB-α, treatment with BMS-345541 was expected to selectively prevent phosphorylation of IκB-α. In fact, the levels of phosphorylated IκB-α decreased significantly in drug-treated cells (Figure 3). Interestingly, the levels of phosphorylated Akt also decreased; whether this unexpected result is physiologically relevant or due to nonspecific activity of the inhibitor or experimental error remains to be determined.

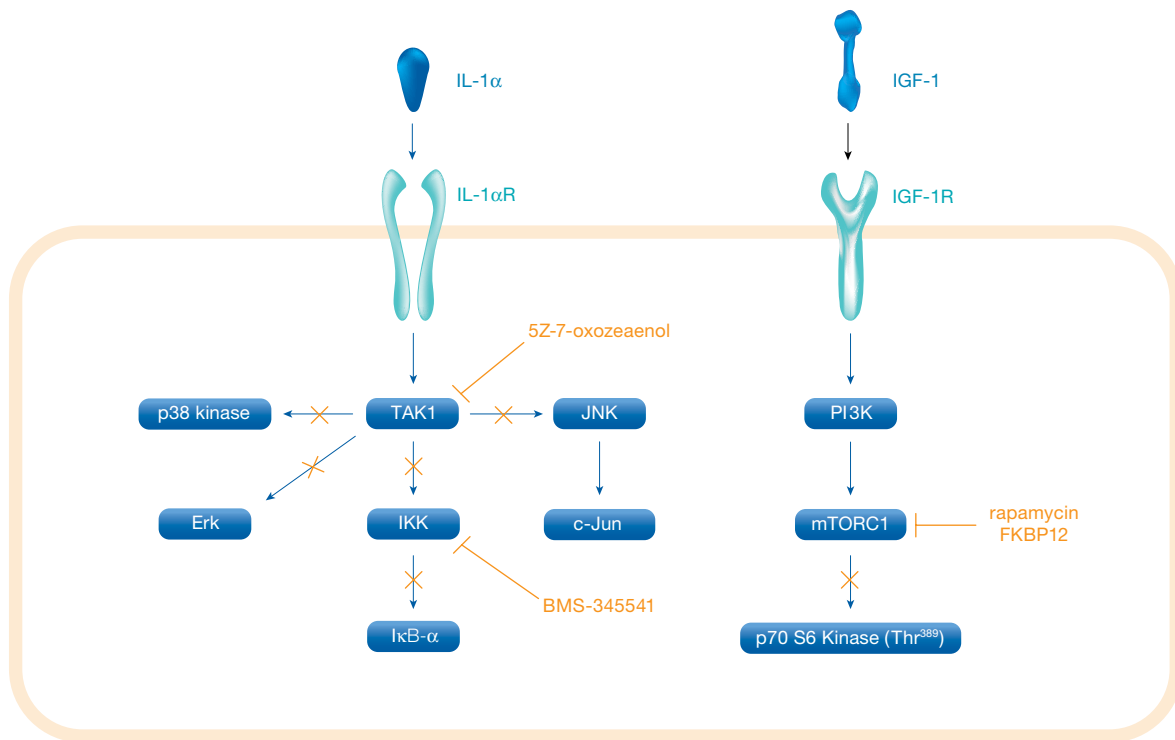


Fig. 1. Schematic representation of signaling pathways in HepG2 cells. IL-1 α signal transduction is inhibited by 5Z-7-oxozeaenol and BMS-345541; rapamycin inhibits IGF-1 signal transduction.

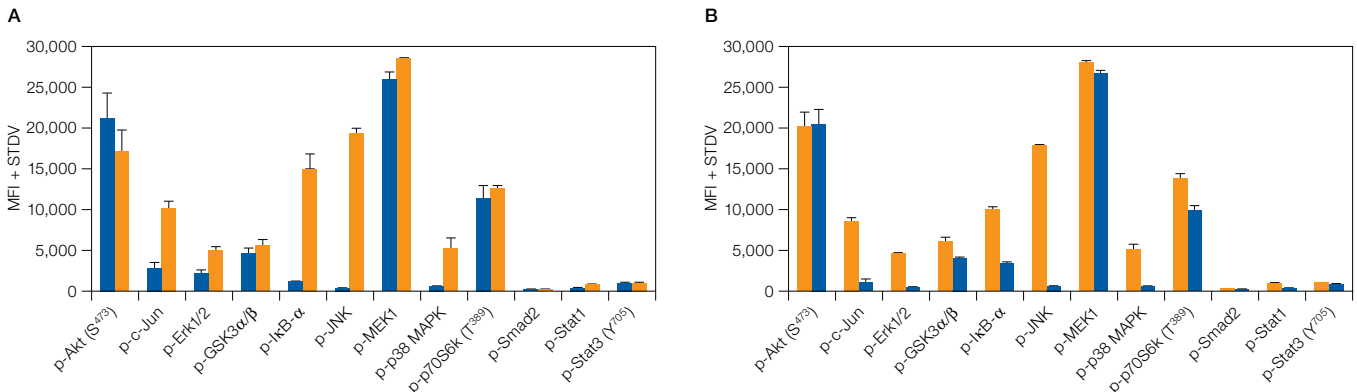


Fig. 2. Elucidation of the IL-1 α signaling pathway in HepG2 cells. **A**, Cells were treated with IL-1 α for 30 min, harvested, and tested with a 12-plex assay; untreated (■); IL-1 α (■). **B**, cells were pretreated with 4 μ M 5Z-7-oxozeaenol for 30 min and then stimulated with IL-1 α for 30 min. pretreated with 5Z-7 (■); IL-1 α (■).

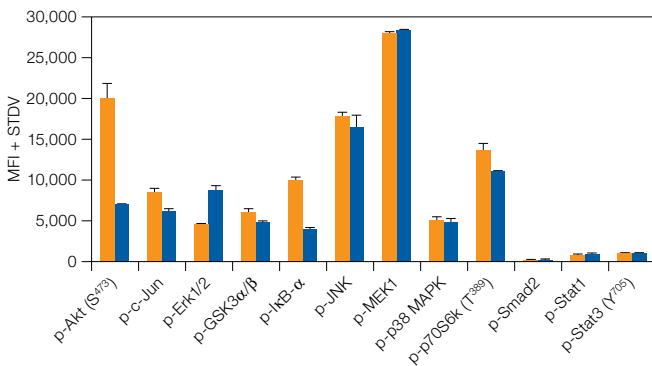


Fig. 3. The effect of BMS-345541 on IKK. Cells were treated with IL-1 α for 30 min or pretreated with 28 mM BMS-345541 for 30 min and then stimulated with IL-1 α for 30 min. pretreated with BMS-345541 (■); IL-1 α (■).

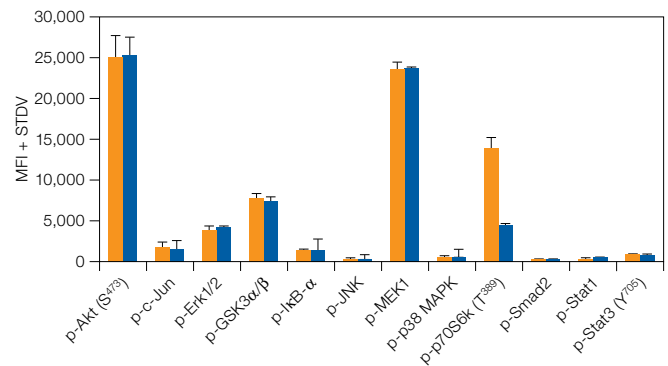


Fig. 4. The effect of rapamycin on IGF-1 signal transduction. Cells were treated with IGF-1 for 30 min, or pretreated with 35 nM rapamycin for 30 min and then stimulated with IGF-1 for 30 min. pretreated with rapamycin (■); IGF-1 (■).

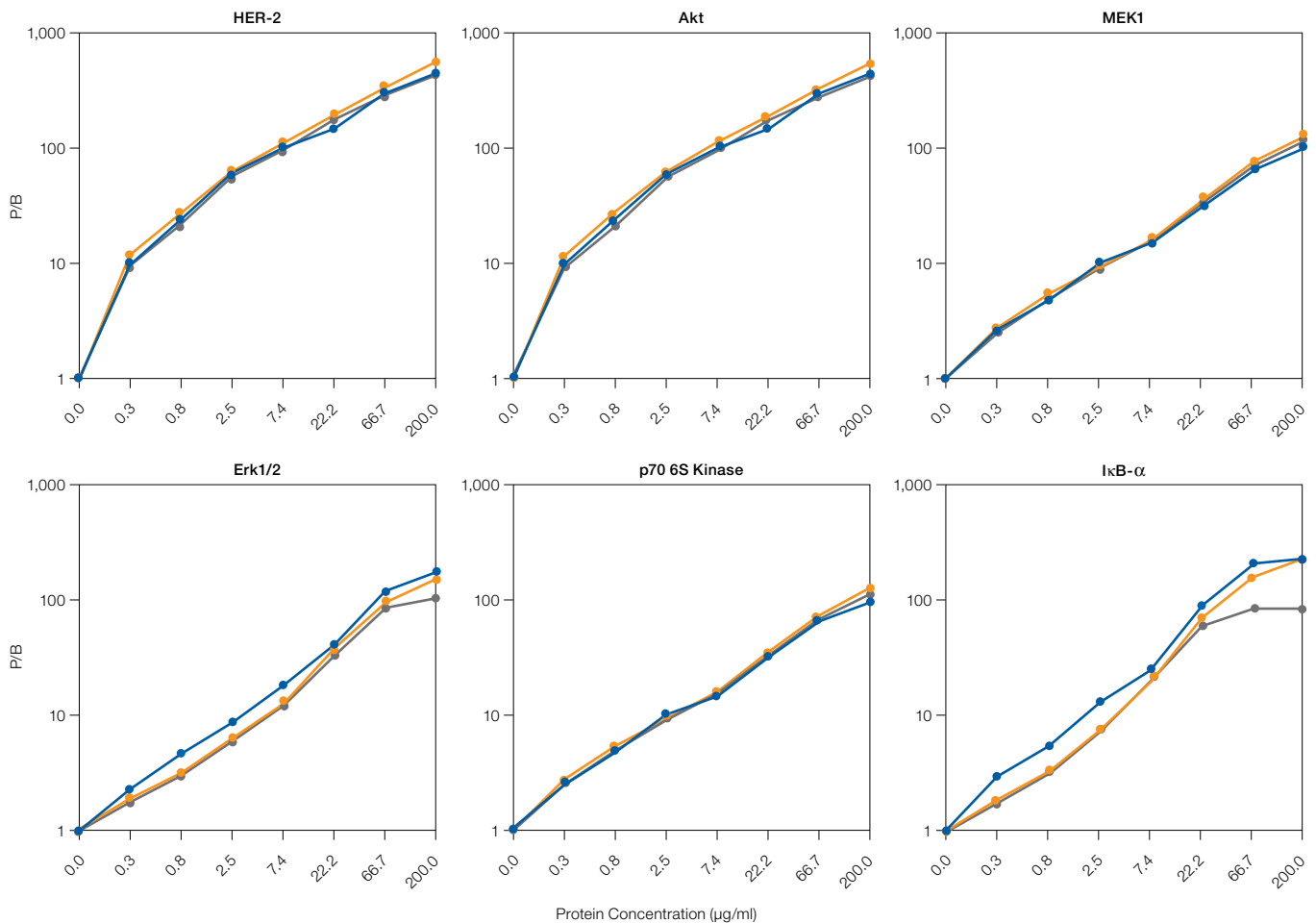


Fig. 5. Similar performance across all three Bio-Plex systems. An 11-plex panel of total cell signaling protein assays was tested with serially diluted SK-BR-3 cell lysate on the three instruments: the Bio-Plex MAGPIX (—○—), the Bio-Plex 200 (—■—), and the Bio-Plex 3D (—▲—) systems, and the P/B ratios were plotted vs. protein concentration. The results for six representative targets are presented here.

Third, we stimulated cells with IGF-1 in the presence of rapamycin (35 nM), a specific inhibitor of the mTOR pathway. Rapamycin, also known as sirolimus, works by binding FKBP12 to form a complex that directly binds to mTORC1 and prevents further phosphorylation of p70S6k, 4E-BP1, and, indirectly, other proteins involved in transcription, translation, and cell cycle control (Vignot et al. 2005). As expected, of the 12 assays tested, only phosphorylated p70S6k levels were reduced. This data further demonstrates the specificity of the Bio-Plex Pro cell signaling assays (Figure 4).

It should be noted that all of these studies including drug effects, cytokine treatments, and time courses were performed by one investigator in less than 24 hours using less than two full assay plates. For comparison, the same study performed by western blotting would require 12 blots for phosphoprotein and 12 blots for total protein assays, requiring a period of at least three weeks, excluding time for band quantification. These studies confirmed the exceptional sensitivity, high specificity, and ease of use of the Bio-Plex Pro cell signaling assays in mapping intricate pathway interactions.

Similar Performance across Three Bio-Plex Platforms

Bio-Rad produces three Bio-Plex systems utilizing xMAP magnetic bead technology: the Bio-Plex 200, Bio-Plex 3D, and Bio-Plex[®] MAGPIX[™] multiplex readers. To compare the performance of the three platforms, we tested an 11-plex panel of total cell signaling protein assays with serially diluted SK-BR-3 cell lysates using each instrument. The data for the Bio-Plex MAGPIX, Bio-Plex 200, and Bio-Plex 3D systems were acquired using the default, high PMT, and enhanced high PMT settings, respectively. The experiments were run independently on the same day, and the lysate-to-buffer (P/B) MFI ratios were calculated. All 11 assays yielded clear detection on all three instruments at a protein lysate concentration as low as 2 μg/ml, with dilution curves displaying similar shapes and P/B ratios (Figure 5). Thus, the Bio-Plex Pro cell signaling assays are portable across the three instrument platforms, with most assays exhibiting ~100-fold increased signal at a protein concentration of 200 μg/ml (10 μg/test) over the blank. This result was expected and is consistent with the testing of other Bio-Plex panels (Zimmerman et al. 2012).

Conclusions

The increasing use of Bio-Plex platforms in both academia and the pharmaceutical industry in recent years has moved this technology into the mainstream. The newly configured Bio-Plex Pro cell signaling magnetic bead assays were designed to streamline assay processing and are compatible with all three platforms. Assay precision, specificity, and sensitivity were improved over the polystyrene (non-magnetic) bead format and were extensively evaluated according to standard industry guidelines. The validation and application studies described herein demonstrate the robustness of these assays and their advantages for proteomics researchers. By measuring multiple markers simultaneously, these cell signaling assays reduce time, cost, and sample volume compared with more traditional methods such as ELISA and western blotting.

References

- Alexopoulos LG et al. (2010). Networks inferred from biochemical data reveal profound differences in toll-like receptor and inflammatory signaling between normal and transformed hepatocytes. *Mol Cell Proteomics* 9:1849–1865.
- Boulanger et al. (2001). Neuronal plasticity and cellular immunity: shared molecular mechanisms. *Current Opinion in Neurobiology* 11:568–578.
- Burke et al. (2003). BMS-345541 is a highly selective inhibitor of I κ B kinase that binds at an allosteric site of the enzyme and blocks NF- κ B-dependent transcription in mice. *JBC* 278:1450-1456.
- Chang L and Karin M (2001). Mammalian MAP kinase signaling cascades. *Nature* 410:37-40.
- Manning G et al. (2002). The protein kinase complement of the human genome. *Science* 298:1912–34.
- Ninomiya-Tsuji J et al. (2003). A resorcylic acid lactone, 5Z-7-oxozeanol, prevents inflammation by inhibiting the catalytic activity of TAK1 MAPK kinase. *JBC* 278:18485-18490.
- Vignot S et al. (2005). mTOR-targeted therapy of cancer with rapamycin derivatives. *Annals of Oncology* 16:525–537.
- Yates D (2011). Development: Directing development through phosphorylation. *Nature Reviews Neuroscience* 12:248-249.
- Yeung D et al. (2011). Multiplex analysis of rat cytokines and diabetes biomarkers using Bio-Plex Pro™ rat cytokine and diabetes assays. *Bio-Rad Bulletin* 6119.
- Zimmerman R et al. (2012). Development and validation of Bio-Plex Pro™ human cancer biomarker panel 2. *Bio-Rad Bulletin* 6304.
- Zhou H and Geng W (2011). Analysis of murine Th17 cytokine profiles using the Bio-Plex Pro™ mouse Th17 panel. *Bio-Rad Bulletin* 6118.

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