Comparison of Bio-Plex® Assays to Immunohistochemistry (IHC) for Measuring Phosphorylated Proteins in an *In Vitro* and *In Vivo* Model of Lung Cancer

Kathleen Rogers, Katherine Crosby, Jeremy Fisher, Michael Lewis, Bradley L. Smith and Reginaldo Prioli • Cell Signaling Technology, 3 Trask Lane, Danvers, MA 01923

Abstract

Protein phosphorylation is a regulatory mechanism for cell growth, survival, apoptosis and response to extracellular signaling events. Deregulation of these pathways is central to the onset and progression of cancer. Identification of proteomic biomarkers is crucial to the understanding and management of cancer. Therefore there is a great need to develop sensitive, quantifiable and multiplexable assays to measure the phosphorylation state of these proteins. Currently, in the clinical setting IHC is the preferred method for the detection of phosphoproteins in cancerous solid tumor tissues. However, this technique is constrained by limited throughput, limited ability to multiplex, lack of quantification and the need for specialists to interpret the results. An alternative technology is a multiplexable bead-based ELISA, such as the Bio-Plex® assay. The objective of this study was to demonstrate substantial equivalence between IHC and Bio-Plex® detection of phosphorylated biomarkers in an *in vitro* and *in vivo* model of cancer. To demonstrate such equivalency, we used HCC827 cells, a non-small cell lung cancer line (NSCLC). Cells or xenografts in nude mice were treated with Gefitinib to determine the capability of IHC or Bio-Plex® assays to detect drug affects on the target and on downstream signaling. The effect of Gefitinib inhibition *in vitro* and *in vivo* was measured by determining the degree of phosphorylation of Akt, Erk, S6 ribosomal protein and EGFR by Bio-Plex® and IHC. To compare treatment groups, phosphorylation ratios were determined (phosphoprotein value/ total protein value) for each analyte. Comparisons of Gefitinib-treated and control samples for both the *in vitro* cell cultures and the *in vivo* xenografts yielded similar results. Both Bio-Plex® and IHC assays detected reduced phosphorylation of Erk, S6 ribosomal protein and EGFR in HCC827 cells or xenografts treated with Gefitinib compared to control samples. These results demonstrate that the Bio-Plex® assay is substantially equivalent to IHC analysis. In summary, the Bio-Plex® assay offers a relatively simple way to quantify multiplexed proteomic biomarkers using small sample volumes from cell lysate or tumor tissue. Based on these results, Bio-Plex® is a desirable method for detection of the phosphoprotein status of multiple biomarkers.

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Introduction

Receptor tyrosine kinases (RTKs) mediate growth, differentiation and developmental signals in cells by adding phosphate groups to substrate proteins to change their activation state. When RTKs are altered or mutated they can become potent disease drivers, causing the initiation and progression of a number of cancers. To effectively identify such disease drivers and treat patients, clinicians need to understand the degree of activation of a particular pathway and the engagement with downstream components in order to identify the best treatment for each patient.

In order to efficiently identify patients whom would be most likely to benefit from targeted drug therapy, it is essential to develop new laboratory techniques. Standard laboratory and clinical assays such as immunohistochemistry (IHC), ELISAs and Western blots are limited in by the fact they can only detect expression of a limited number of proteins at once. In contrast, the Bio-Plex® (Luminex) bead-based ELISA platform allows the opportunity to develop assays to detect cell signaling events that may assess up to 100 protein targets from a single sample. In a partnership, Cell Signaling Technology and Bio-Rad Laboratories developed, optimized and validated Bio-Plex® assays to detect and measure therapeutic targets and determinants of therapeutic efficacy. The objective of this study was to determine if the Bio-Plex® assay was equivalent or better than IHC and to determine if results obtained *in vitro* could be reproduced *in vivo* in a non-small cell lung cancer line HCC827.

Methods

Lysate preparation

- For the *in vitro* study, HCC827 cells (non-small cell lung cancer) were treated with 100ng/ml of EGF or inhibited with 1mM Gefitinib (Astra-Zeneca), then stimulated with EGF. Untreated cells were used as controls
- For the *in vivo* study, 10^7 HCC827 cells were injected subcutaneously into Nude mice (Taconic)
- When the tumors were 1 cm^3, 5 mice were administered vehicle control (100 ul Tween-80) and 5 mice were administered 150 mg/kg of Gefitinib dissolved in Tween-80
- Tumors harvested 24 hr after treatment and flash frozen
- 30mg of tissue was removed from each sample, placed in Bio-Plex® cell lysis buffer and homogenized by mechanical lysis
- Cells lysed with Bio-Plex® cell lysis buffer (Bio-Rad®), and protein concentration measured

Bio-Plex®

- Bio-Plex® assays (Bio-Rad) were used to evaluated levels of total and phospho-EGFR, Erk, S6 Ribosomal Protein and Akt
- Antibodies against specific targets were coupled to dyed beads and samples added with beads to the wells of a 96-well plate Biotin-labeled detection antibodies were added to each well
- Beads were detected with fluorescently labeled avidin. Samples were analyzed with the Bio-Plex® Suspension Array System

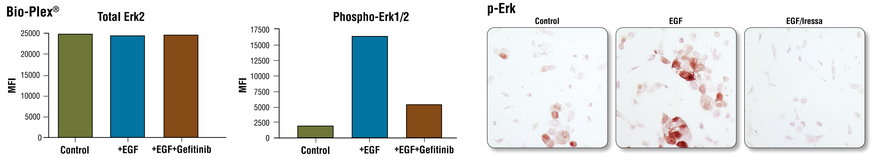
Immunohistochemistry and Immunocytochemistry

- Cell and tumor samples cut to 4 μm thickness, deparaffined, and rehydrated
- Antigen retrieval performed in 10mM citrate/Tris buffered saline, pH 6.0
- Slides washed in deionized water then incubated in 3% hydrogen peroxide
- Sections blocked in 5% normal goat serum for 1 hr
- Slides incubated over night at 4°C with appropriate primary antibodies
- Anti-rabbit secondary antibody (Vector) added. Vector ABC elite kit reagent used for detection
- Samples quantified using Axiovision Image Analysis (cell pellets) or scored by a veterinary pathologist (xenografts)

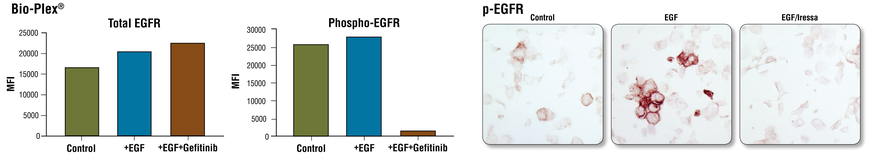
Results

Cell Lysate

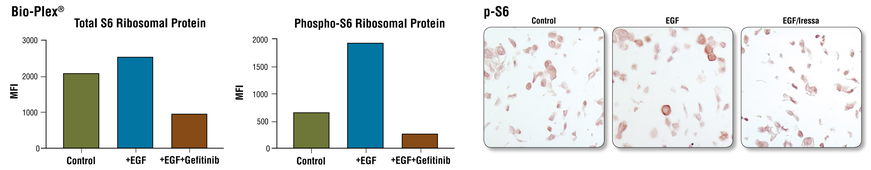
Erk



EGFR



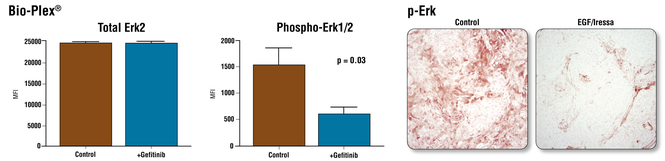
S6 Ribosomal Protein



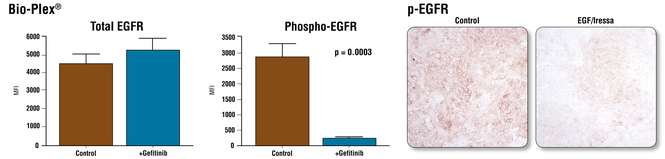
** Akt results did not show differences (data not shown).*

Xenograft

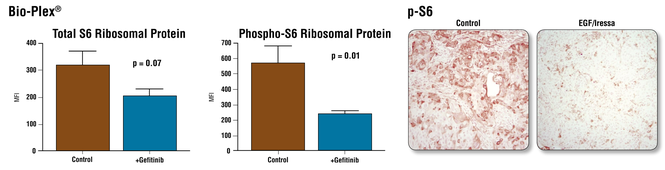
Erk



EGFR



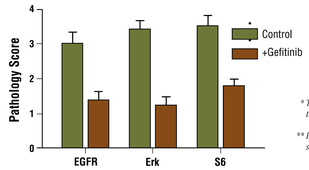
S6 Ribosomal Protein



Use or phosphorylation index (PI=[phospho-protein Bio-Plex® MFI/ total protein Bio-Plex® MFI]*100) confirms equivalence of *in vitro* and *in vivo* data.

	Control Cell Lysate	Cell Lysate Gefitinib-treated	Fold Decrease of index *in vitro*	Control Xenograft	Gefitinib-treated Xenograft	Fold Decrease of index *in vivo*
S6 Ribosomal Protein	76.5	28.3	2.7	179.9	119.4	1.5
EGFR	387.3	25.1	15.4	63.2	4.2	15.0
Erk	67.3	22.1	3.0	6.3	2.5	2.5

IHC Analysis



** There was a significant difference ($p < 0.05$) between control and Gefitinib treated mice for each phosphoprotein measured.*

*** Pearson correlation analysis was performed and there was a statistically significant correlation between Bio-Plex® and IHC ($p = 0.01$).*

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Conclusions

- Bio-Plex® assay offers a simple way to quantify multiplexed proteomic biomarkers using small sample volumes from cell lysate and tumor tissue.
- Gefitinib treatment resulted in decreased phosphorylation of S6 Ribosomal Protein, EGFR and Erk both *in vitro* and *in vivo* detected by Bio-Plex® and ICC/IHC.
- Bio-Plex® assay can be used to detect phospho-proteins from cell lysate and tissue samples and suggest that the Bio-Plex® assay would be applicable for use in clinical samples.
- **The results obtained by Bio-Plex® and IHC analysis of tumor tissue correlate statistically and are substantially equivalent.**

BIO-RAD *CST and Bio-Rad have an alliance in which CST's expertise is applied to screen and validate capture and detection antibody pairs to expedite Bio-Plex® assay development.*