Comparison of Protein Phosphorylation in Cell Line and Xenograft Samples by Bio-Plex® and Western Blot Techniques.

Kathleen Rogers, Jeremy Fisher, Michael Lewis, Bradley L. Smith and Reginaldo Prioli
Cell Signaling Technology Inc., Danvers, MA USA

Introduction

Receptor tyrosine kinases (RTK) mediate growth, differentiation and developmental signals in cells by adding phosphate groups to substrate proteins to change the activation state of the protein. When RTKs are altered or mutated they can become potent oncogenes, causing the initiation and progression of a number of cancers. These pathways play a key role in the development new drug therapies. In addition, to effectively diagnose and treat patients, clinicians need to understand the degree of activation of a particular pathway and the engagement with downstream components in order to target a set of interconnected kinase-driven events along a signaling pathway. This will enable efficacious targeting of treatment.

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 optimize the Bio-Plex® assay using *in vitro* and *in vivo* samples of the non-small cell lung cancer line HCC827 and to compare the results to Western blots.

Methods

***In vitro* study:**

- HCC827 cells (non-small cell lung cancer) were treated with 100ng/ml of EGF or inhibited with 1mM Iressa (Astra-Zeneca), then stimulated with EGF. Untreated cells were used as controls.
- Cells were lysed with Bio-Plex cell lysis buffer (Bio-Rad), and protein concentration measured.

***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 (100ul Tween-80) and 5 mice were administered 3.75mg of Iressa (Astra-Zeneca) dissolved in Tween-80.
- Tumors harvested 24h after treatment.
- 30 mg of tissue was removed from each sample, placed in Bio-Plex® cell lysis buffer, homogenized by mechanical lysis using the Qiagen Tissue Lyser and protein concentration measured.

Both studies:

- Bio-Plex® assays were used to evaluate levels of Total and phospho-Akt, Erk, S6 ribosomal protein and EGFR.
- For comparison, Western blot assays were used to evaluate the same targets and quantified using Scion Imaging Software (Scion Corp.).

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.

Results

Phosphorylation ratios of HCC827 cell and xenograft lysates.

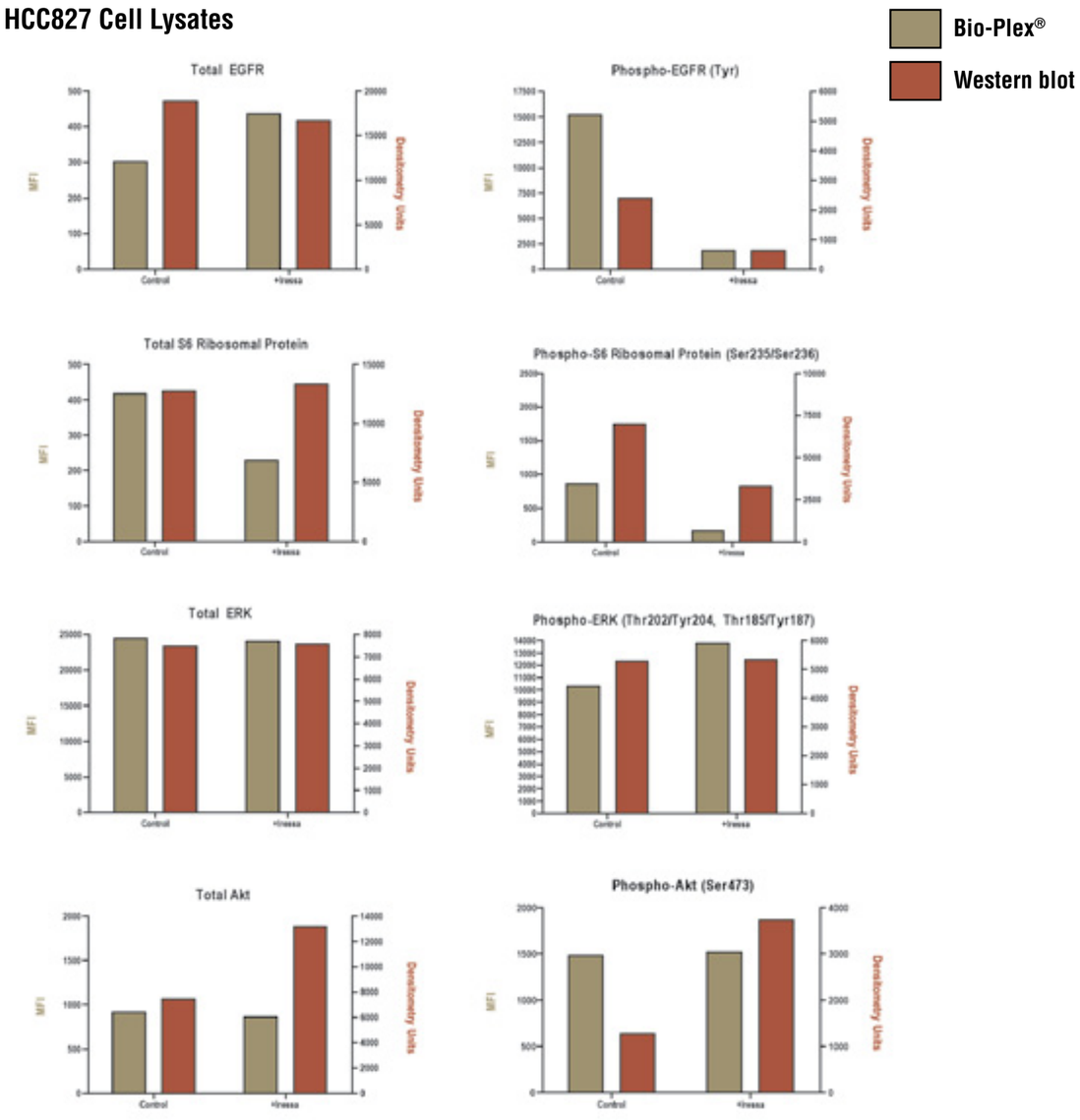
	Control cells	Iressa-Treated cells	Fold decrease in ratio *in vitro*	Control Xenograft	Iressa-Treated Xenograft	Fold decrease in ratio *in vitro*
S6 Ribosomal Protein	206.8	70.2	2.9	179.9	115.4	1.56
EGFR	5034.4	4.17	1207.2	1129.0	88.1	12.8
Erk	42.0	57.2	--	6.3	2.5	2.5
Akt	1.6	1.7	--	51.7	39.6	1.3

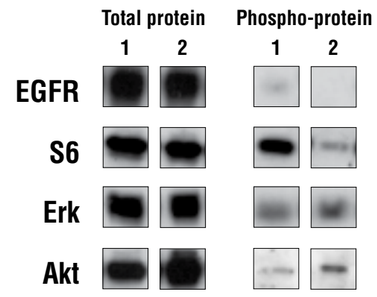
The phospho-ratios of control and Iressa-treated cell lysates and of control and Iressa treated xenograft lysates for each analyte were compared. Phospho-ratios = (phospho-protein Bio-Plex® MFI/total protein Bio-Plex® MFI)(100). Iressa decreased phosphorylation of S6 ribosomal protein and EGFR in treated HCC827 cells, as measured by Bio-Plex®. The phosphorylation of S6 ribosomal protein, EGFR, Erk and Akt for Iressa-treated xenografts was decreased compared to controls.

Fold-reduction in phosphorylation of analytes after Iressa treatment compared to controls.

	Cell Lysates (Control vs. Iressa treated)		Xenograft Lysates (Control vs. Iressa treated)	
	Bio-plex®	Western	Bio-plex®	Western
pS6	5.3	2.1	2.3	2.3
pEGFR	8.3	3.8	12.9	2.8
pErk	--	--	2.5	3.3
pAkt	--	--	2.4	3.7

HCC827 Cell Lysates

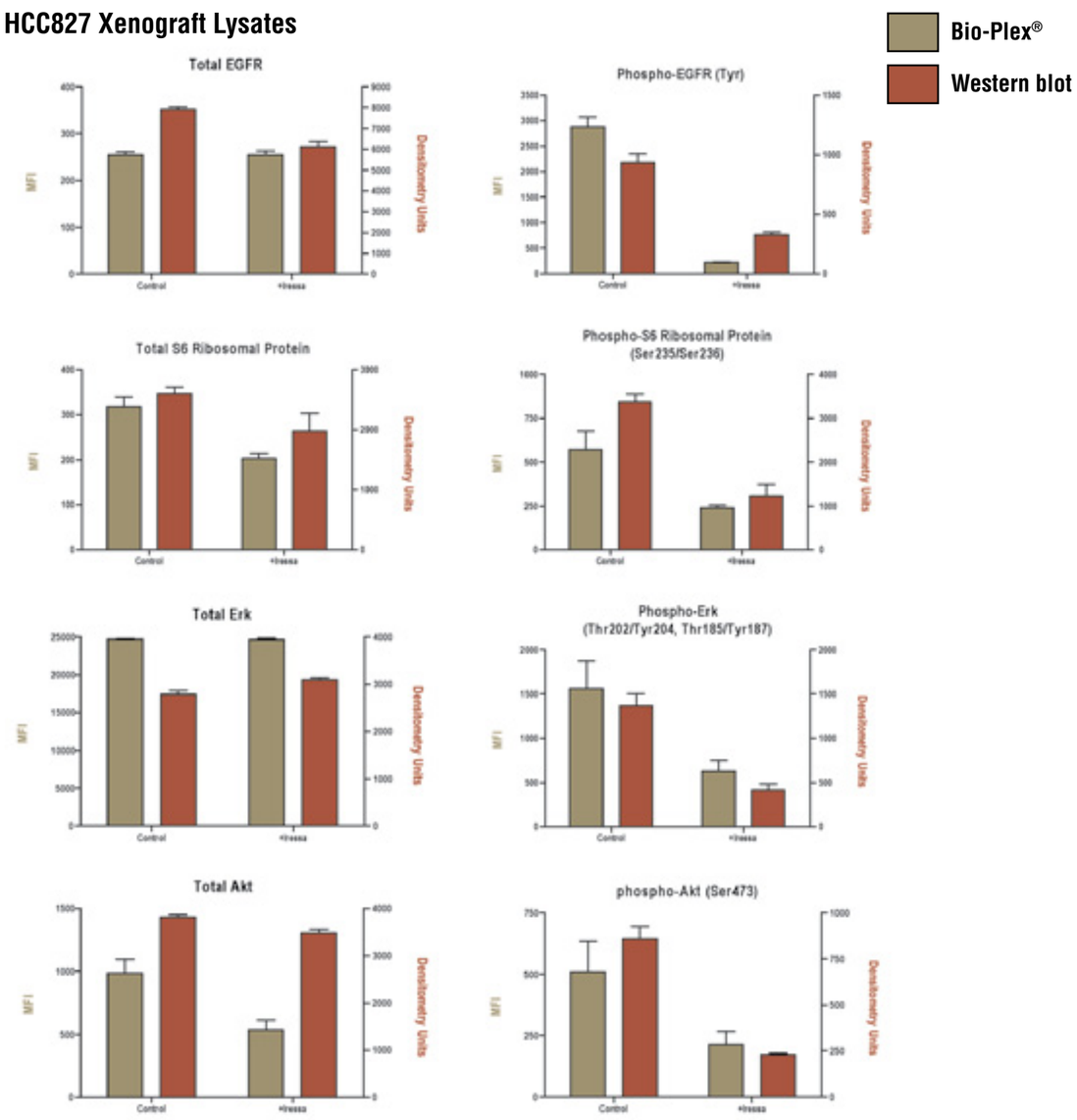


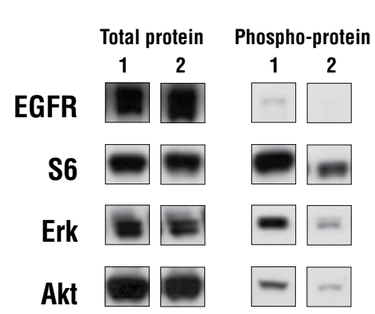


Western blot analysis of HCC827 cell lysate (above). Lane 1= HCC827 control cell lysate, Lane 2= HCC827 cells inhibited for 2 h with 1mM Iressa. Proteins were transferred onto nitrocellulose membrane and probed with antibodies specific for total and phospho-Akt, Erk (p42/44), S6 ribosomal protein and EGFR (Cell Signaling Technology).

Comparison of Bio-Plex® and Western Blot results for the HCC827 cell lysates (left). The mean fluorescent intensity (MFI) from the Bio-Plex® assays was compared with the densitometry units (mean number of pixels per peak) of the western blot analyses for each of the analytes tested.

HCC827 Xenograft Lysates





Western blot analysis of HCC827 xenograft lysate (above). Lane 1= control mouse xenograft lysate, Lane 2= xenograft lysate from mouse treated with 3.75mg Iressa for 24 h.

Comparison of Bio-Plex® and Western Blot results for the HCC827 xenograft lysates (left). The mean fluorescent intensity (MFI) from the Bio-Plex® assays was compared with the densitometry units (mean number of pixels per peak) of the western blot analyses for each of the analytes tested. Overall, both the Bio-Plex® and western blot results followed similar trends.

Conclusions

- The Bio-Plex® assay can be used for both cell lysate and tissue homogenate samples. **To our knowledge, this is the first report using a bead-based ELISA, such as the Bio-Plex® assay, to detect phospho-proteins in tissue samples.**
- Iressa treatment resulted in decreased phosphorylation of S6 ribosomal protein, EGFR *in vitro* and *in vivo* and additionally decreased phospho-Erk and Akt *in vivo*.
- The results obtained by Bio-Plex® and Western blot are comparable.

Cell Signaling TECHNOLOGY®

www.cellsignal.com