SPOTLIGHT feature

Proteomics, Genomics & Microarrays

Use of Quantitative, Multiplex Immunoassays for Improved Exploration of EGFR Signalling

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The ErbB or epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases consists of four members: EGFR/ErbB1/HER1, ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4. The ErbB receptors play crucial roles in propagating signals regulating cell proliferation, differentiation, motility, and apoptosis, contributing to pathological processes such as cancer. The ability to analyse the phosphorylation status of ErbB family members, as well as the phosphorylation status of receptor-related intracellular signal transduction proteins, is necessary for a thorough understanding of this signalling pathway.

To examine the phosphorylation status of the ErbB family and receptor–related intracellular proteins, several assay platforms are available. The Western blot has been the gold standard used to study pathway signalling. Other platforms include ELISA, reverse phase arrays, high content analysis and mass spectroscopy. Although some of these platforms yield absolute quantitative data, the assay is either limited to measuring only one analyte at a time, or there is a high level of difficulty and expense.

This article will describe a quantitative approach to multiplex immunoassays that simultaneously measures both total protein abundance and site-specific phosphorylation at the peptide level with picomolar sensitivity. Data showing use of this technology for exploration of signalling pathways affected by epidermal growth factor receptor inhibition are presented.

The MILLIPLEX® MAP EpiQuant™ EGFR pathway panel profiles 22 ErbB signalling pathway constituents. The assay enables for the analysis and quantitation of tyrosine phosphorylation sites on ErbB2 and ErbB3, multiple sites on ErbB1, as well as tyrosine phosphorylation sites for 16 other receptor related proteins, including ERK1/2 and multiple Gab2 and FAK sites. In addition to containing 20 phosphorylated targets, the assay is also able to quantify total EGFR and a loading control (TAFII68) simultaneously in a single assay well. The EGFR pathway panel enables the detection of phosphorylation events for all panel analytes in dose response and time course experiments.

The EpiQuant™ panel is designed for use on the Luminex® xMAP® system (Luminex Corporation).The platform consists of fluorescently dye-labelled magnetic microspheres, a flow cytometry- or CCD camera-based instrument, and software for data acquisition and analysis. With the xMAP® platform, sandwich assays are performed on a bead. Each bead contains two red dyes; ten different concentrations of each dye yield 100 different possible combinations. Since the beads are in solution rather than fixed to a plate, up to 50 different beads with different capture antibodies can be used with one sample yielding results for up to 50 different proteins. When the beads pass through the reader or chamber, the ratio of the two dyes indicates the bead number.

Streptavidin-phycoerythrin, which fluoresces green, is used as the common detection reagent binding to biotinylated antibodies. The detection system reads red and green thus providing identification of the analyte and quantitation of the amount bound to the bead.

Based on the Luminex® xMAP® platform, EpiQuant™ technology uses antibodies against defined protein sequences to capture peptides resulting from protein linearisation and digestion in a single cell lysate sample. The fragments generated are similar to those generated using mass spectroscopy. *(Figure 1)*. These peptides are then used to generate standard curves, which results in quantitative measurements with picomolar sensitivity.

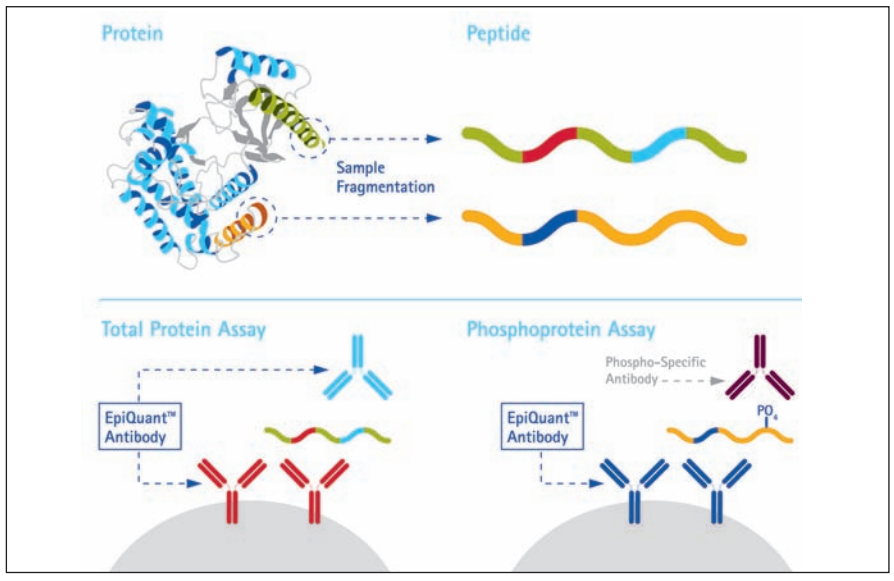

Figure 1. EpiQuant™ technology uses antibodies against defined protein sequences to capture peptides resulting from protein linearisation and digestion in a single cell lysate sample.

This multiplexing technology provides faster answers to cell signalling questions compared to traditional Western blots, mass spectrometry analyses and radioactive phosphorylation assays requiring large amounts of resources, time and sample.

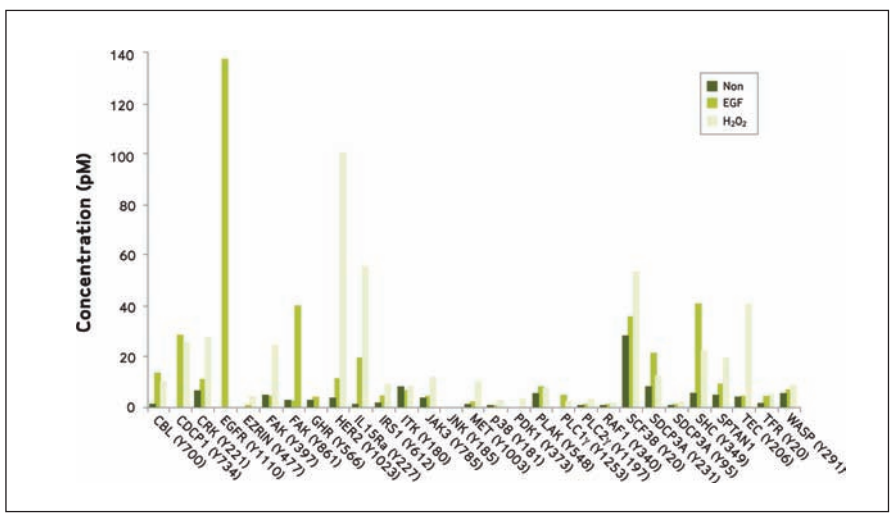

Figure 2. Analysis of A431 human epithelial carcinoma cells treated with EGF, H_2O_2 or vehicle.

Exploring the EGFR Pathway

Figure 2 shows results of an analysis of A431 human epithelial carcinoma cells treated with EGF, H_2O_2, or vehicle. Following lysis and digestion, EpiQuant™ beads (a 22-plex) were added to samples containing the target proteins shown. Samples were analysed on a Luminex® 200™ reader. Data shown are the mean pM values from four observations. Increased phosphorylation was noted for EGFR, Her2, IL-15R, Shc and Tec compared to vehicle-treated cells.

Figure 3 illustrates the results of the dose response experiments for several targets. Of interest is the observation that concentration of EGF needed for maximal activation of the two EGFR phosphorylation sites appears dissimilar.

Figure 4 illustrates the results of the time course experiments. The perpetuation of maximal signal is observed for many targets (ERK1/2 and EGFR, for example) across the whole of the time course, while for other targets (FRS2 and PKCμ, for example) maximal signal levels are observed at the 5-minute time point with a return to basal levels by 20 to 30 minutes.

As would be expected due to receptor internalisation, it appears as though total EGFR levels drop after stimulation, with a rebound to less than resting levels at the one hour time point.

Finally, *Figure 5* illustrates the effect of gefitinib treatment on phosphorylation levels for several targets. Gefitinib is an EGFR-selective inhibitor and chemotherapeutic agent, marketed under the name of Iressa® (AstraZeneca) and is indicated in Europe for the treatment of non-small cell lung cancer (NSCLC). A dose-dependent response is observed in phosphoprotein levels, while total EGFR concentrations remain relatively constant.

Takeshi Shimamura, PhD of Loyola University is using the EpiQuant™ panel to study signaling pathways in NSCLC. The leading cause of cancer deaths in men and women, lung cancer, is now being classified by the oncogenes that are mutated, amplified, or have translocations. These changes often drive tumour growth and resist apoptosis. Understanding the oncogene primarily responsible for lung cancer on a patient-by-patient basis will lead to more personalised, targeted treatments.

Central to personalised treatments is the understanding of what signalling changes lead to cell death and which pathways are affected by inhibition of EGFR. Traditional approaches, such as Western blot, are useful if one knows exactly what targets to study; spot blots are useful if only qualitative or semi-quantitative analyses are required.

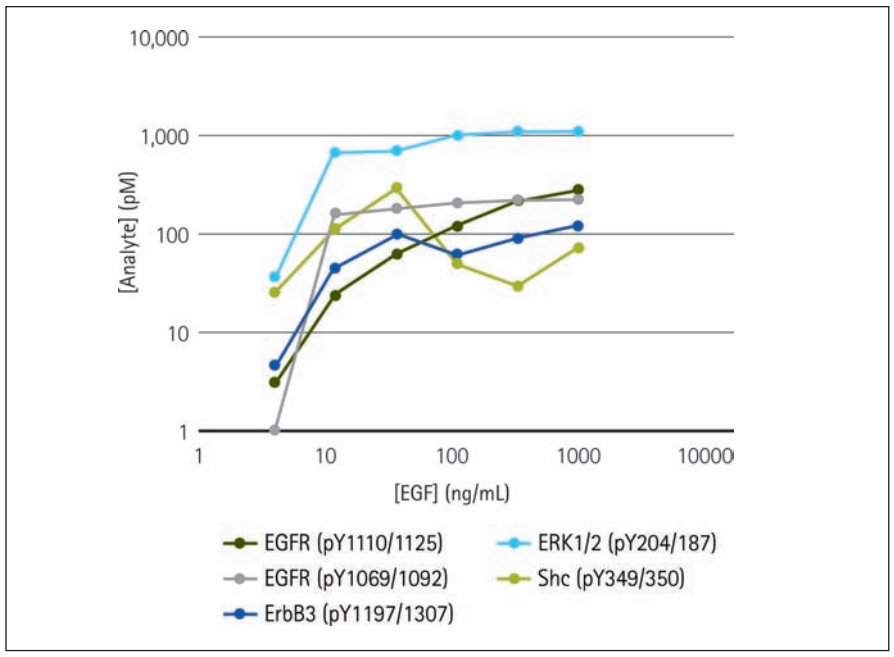

Figure 3. Phosphorylated EGFR pathway proteins, as well as total EGFR, were simultaneously detected in A431 cells treated with 1000, 333, 111, 37,12.3 and 0ng/mL EGF. Lysates were collected after 5 minutes EGF stimulation. Values are internally normalised utilising the TAFII68 loading control.

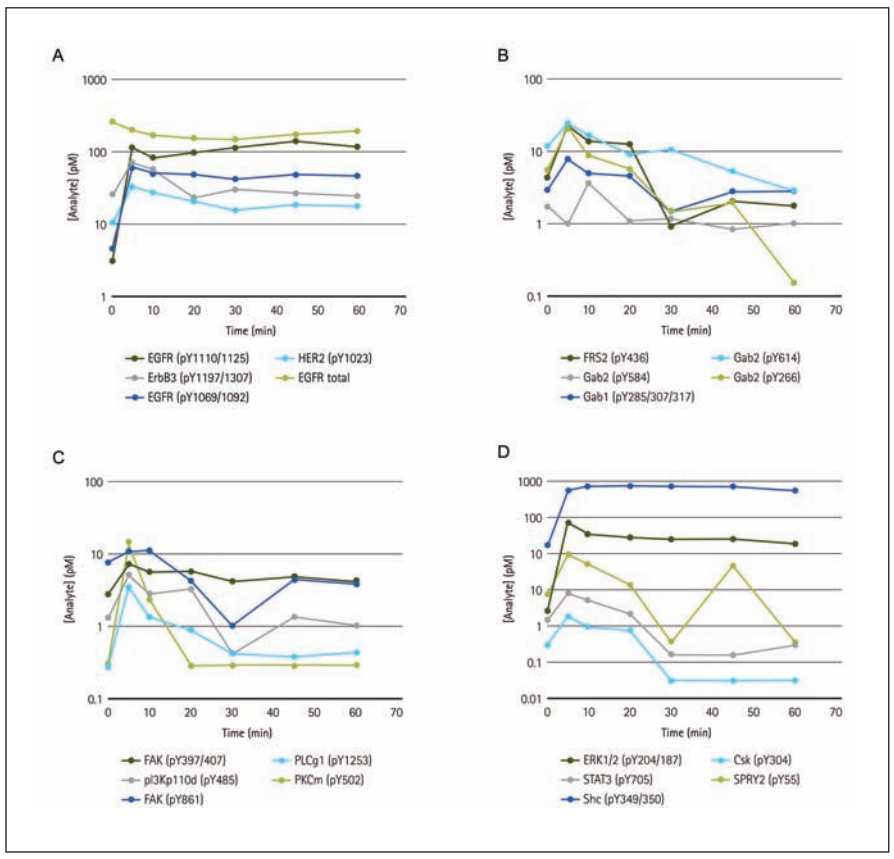

Figure 4. Phosphorylated EGFR pathway proteins, as well as total EGFR, were simultaneously detected in A431 cells treated with 100ng/mL EGF. Lysates were collected at 0, 5, 19, 20, 30, 45 and 60 minutes. Values are internally normalised utilising the TAFII68 loading control.

EpiQuant™ assays can deliver quantitative, in-depth analysis as shown in *Figure 6*. HCC827 NSCLC cells harbouring EGFR exon 19 deletion mutation that sensitise the EGFR to EGFR tyrosine kinase inhibitors (TKIs) were treated with an EGFR TKI, erlotinib (trade name Tarceva®; Genentech/Roche). Data derived from the EpiQuant™ Phosphotyrosine Panel 1 were converted into a heat map (red squares indicate phosphorylation; blue indicates dephosophorylation) to identify signal pathway molecules directly or indirectly activated by EGFR.

In HCC827 cells Shc and Cbl, traditional EGFR downstream molecules, were dephosphorylated (the red squares in the left column change to blue in the erlotinib-treated column). HGFR (c-MET) and VEGFR are likely transphosphorylated by EGFR. The data also indicate that inhibition of mutant EGFR signalling in HCC827 cells also results in dephosphorylation of Crk (pY221/239) and FAK (pY397/407).

The heat map shown in *Figure 7* is derived from the EpiQuant™ EGFR signalling panel which covers more EGFR-related secondary signalling molecules. Data confirm that the inhibition of mutant EGFR in HCC827 cells results in dephoshorylation of ERBB3 as it was reported in literature [1]. EGFR inhibition results in dephosphorylation of MAPK pathway component, ERK (pY204/187), and PI3K pathway adaptor, Gab proteins. Note that EGFR was dephosphorylated at both pY1069/1092 and pY1110/1125 sites upon erlotinib treatment as expected, while there is no significant ($p<0.01$) change in the level of total EGFR protein.

EpiQuant™ multiplex assays are also being used to explore the emergence of gatekeeper mutations that arise in lung cancer patients whose tumours become resistant to EGFR tyrosine kinase inhibitors such as erlotinib and gefitinib. *Figure 8* compares the phosphorylation of signalling proteins in NCI-H1975 cells (resistant to erlotinib and gefitinb but sensitive to irreversible inhibitors such as CL-387,785) and NCI-H1975CLR (the same cell line that has been made resistant to CL-387,785 by culturing in media over 4.5 months with increasing concentrations of CL-387,785).

The heat map reveals that IRS1 and FAK are upregulated in the cells that were made resistant to the irreversible EGFR inhibitor. IGF-1R and IR pathways phosphorylate IRS-1 and activate the P13K pathway. IGF-1R was shown to be active in the NCI-H1975CLR cells and the activity was not influenced by EGFR inhibition [1].

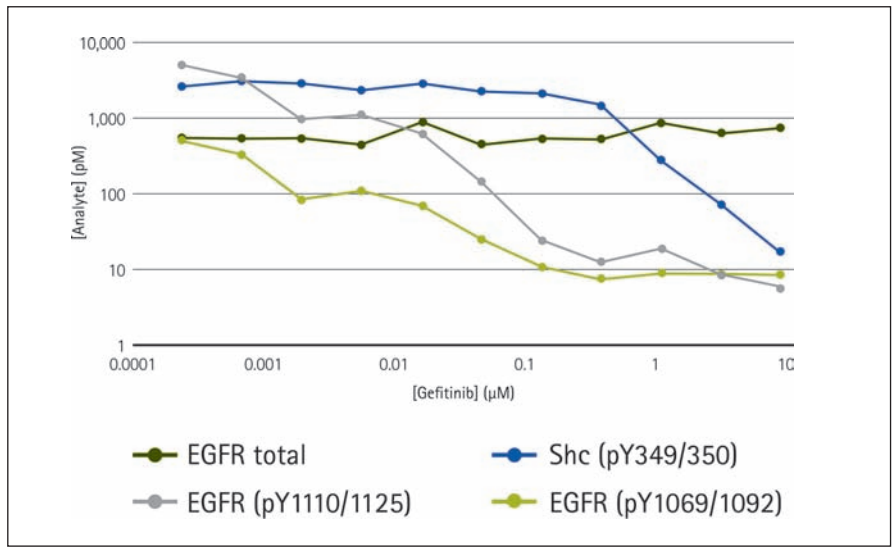

Figure 5. Phosphorylated EGFR pathway proteins, as well as total EGFR, were simultaneously detected in A431 cells treated with gefitinib. Cells were pre-treated with gefitinib 15 minutes prior to the addition of 300ng/mL EGF. Lysates were collected after 5 minutes EGF stimulation. Values are internally normalised utilising the TAFII68 loading control.

Figure 7. (right) Erlotinib treatment promotes dephosphorylation of multiple EGFR pathway proteins in HCC827 cells. The heat map was derived from the EpiQuant™ EGFR signalling panel.

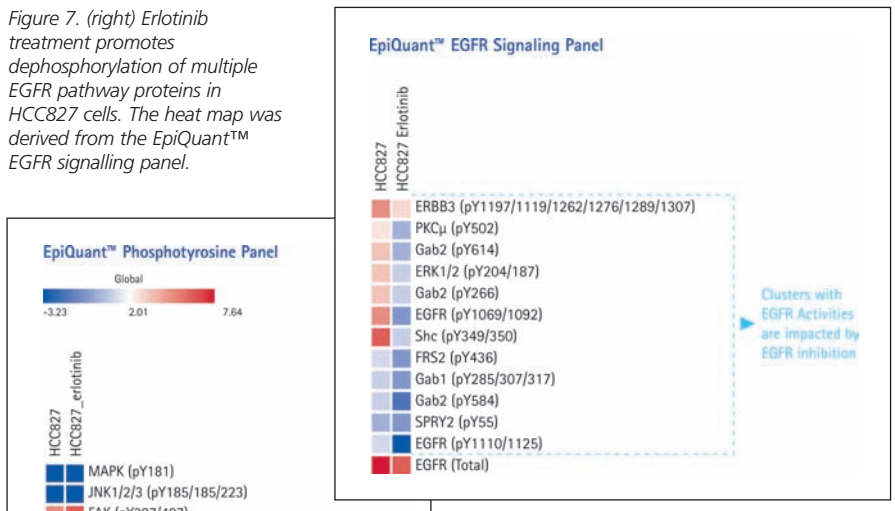

Figure 6. (above) Heat map comparing phosphorylation of signal pathway molecules in HCC827 NSCLC cells following exposure to 1µM erlotinib for 24 hours. Red squares indicate phosphorylation; blue indicates dephosophorylation.

Figure 8. (right) Comparison of phosphorylation of signalling proteins in NCI-H1975 cells (resistant to erlotinib and gefitinb but sensitive to irreversible inhibitors such as CL-387,785) and NCI-H1975CLR (the same cell line that has been made resistant to CL-387,785).

Conclusion

The complexity and number of protein targets involved in signalling events, as well as cellular responses, require tools that enable multiplex analysis of samples. The approach described in this article enables analysis of a greater number of intracellular analytes per well and simultaneous measurement of multiple phosphorylation sites with picomolar sensitivities, absolute quantitation of multiple phosphorylation sites on the same protein, and absolute quantitation of both total and phosphoproteins.

EpiQuant™-based assays can provide greater insight into complex signalling pathways where mutations can lead to cancer and can help identify changes that take place on a patient-by-patient basis.

To learn more about Dr Shimamura's research and the use of EpiQuant™ assays, please visit:

www.millipore.com/life_sciences/flx4/protein_webinar

References

1. Shimamura, T et al. Hsp90 inhibition suppresses mutant EGFR-T790M signaling and overcomes kinase inhibitor resistance. Cancer Res. 2008; 68: 5827-5838.