Memorial Sloan-Kettering Cancer Center

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# Independent Measurement of MEK Phosphoforms by Capillary Immunoassay

Nineveh Parker[1], Christine Pratilas[2], Eric Joseph[2], Poulikos Poulikakos[2], Neal Rosen[2], Erik Gentalen[1].

[1]Cell Biosciences, Inc., 1050 Page Mill Road, Palo Alto, CA 94304

[2] Program in Molecular Pharmacology and Chemistry, Memorial Sloan-Kettering Cancer Center, New York, NY.

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## Abstract

Activation of the MAPK pathway involves a complicated web of MEK phosphorylations. The 2 MEK isoforms are regulated by at least 3 other enzymes – PAK, RAF and ERK. Up until now it has been impossible to quantitate and determine the stoichiometry of the various multiply phosphorylated MEK forms. We have developed a new capillary immunoassay which resolves the different MEK variants, and allows measurement of the relative abundance of each form with a single antibody. This measurement of how the multiply phosphorylated forms change gives insight into how the dynamics of pathway feedback and activity change in response to drug treatment.

We have shown that signaling upstream of MEK kinase is inhibited by negative feedback in tumor cells in which the pathway is driven by HER kinases. In these cells, MEK1 is phosphorylated at ERK-- and PAK-dependent sites (T292, S298), whereas phosphorylation on RAF-dependent sites is undetectable. A selective MEK inhibitor inhibits ERK phosphorylation, relieves the negative feedback and activates MEK phosphorylation in these cells. Under these conditions, phosphorylation of both kinases on the RAF dependent sites (S217, S222) is markedly induced.

Thus, inhibition of MEK/MAPK signaling in these cells abrogates upstream feedback of the pathway and results in a complex change in phosphorylation of MEK due to multiple kinases. The capillary immunoassay allows determination of complex changes in phosphorylation of MEK kinase by PAK, RAF and ERK kinases in response to MEK inhibition. This technique will be useful in mapping pathway network response to targeted drugs *in vitro* and *in vivo*. .

## The Firefly™ Assay

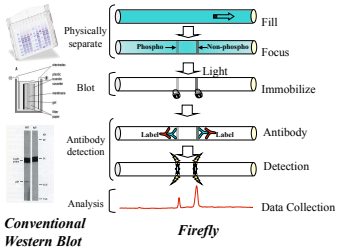


**Figure 1** Assay method. A 5 cm length of 100 m I.D. capillary is filled by touching its tip to a sample droplet as small as 400 nl, containing sample proteins, separation buffer, and internal isoelectric point standards. IEF is used to focus proteins and peptides in 4-30 min. Focused protein bands are immobilized by 15 to 60 sec exposure to a UV light source. Primary antibody specific for the protein of interest may then be flowed through the capillary, binding to focused bands of its target protein. Following washing, secondary antibody is flowed through the channel to bind to the primary antibody. This secondary antibody is labeled with a flurophore and/or a detectable reporter enzyme such as horseradish peroxidase (HRP). The capillary may then be imaged using either a laser induced fluorescence scanner or chemiluminescence. For chemiluminescence detection chemiluminescent reagents are flowed through the capillary, reacting with HRP to generate light, which is detected by a CCD camera imaging the full length of multiple capillaries simultaneously.

## MEK Inhibitor Induces MEK Phosphorylation In SKBR3 But Not In SKMEL28

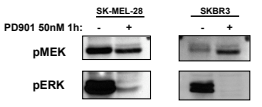


**Figure 2** Western analysis of MEK and ERK phosphorylation in BRAF (V600E) SKMEL28 and HER2 amplified SKBR3 tumor cell culture following MEK inhibition using PD0325901 (Pfizer). ERK phosphorylation is significantly reduced in both cell lines in response to MEK inhibitor. MEK inhibition induces MEK phosphorylation in RTK activated tumor cells, but not in BRAF V600E activated tumor cells.

## Quantitation of ERK Phosphorylation

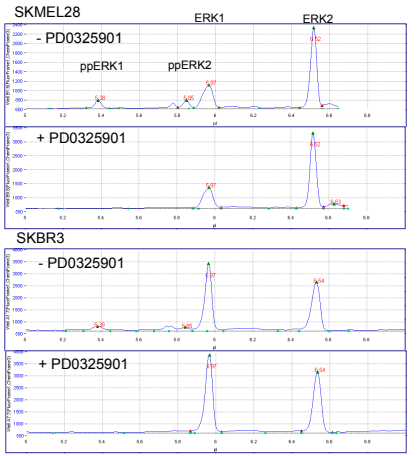


**Figure 3** Firefly analysis of samples using a pan-specific ERK1/2 antibody. ppERK1 and ppERK2 designate dual phosphorylated forms of ERK (T202/Y204). ERK1 and ERK2 designate unphosphorylated forms of the proteins. In control sample of SKMEL28 the dual phosphorylated forms of ERK1 and ERK2 represent 23% and 10% of their total signal, respectively. In the untreated SKBR3 sample 10% of ERK1 and 7% of ERK2 are dual phosphorylated. No dual phosphorylated ERK is detected in either of the PD0325901 treated samples.

ERK peak identification as reported in: O'Neill et al. PNAS 103:44, p 16153

## Firefly Assay Determination Of MEK Phosphorylation At The Raf-specific S217 Phosphorylation Site

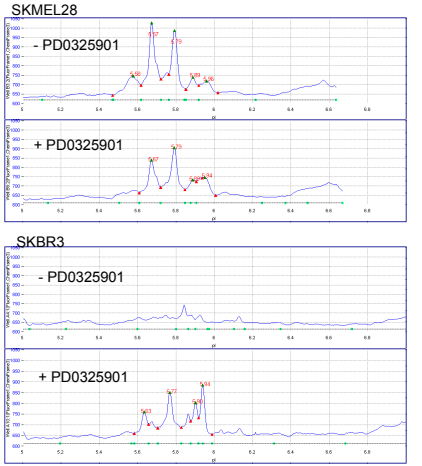


**Figure 4** Firefly analysis of samples using an anti-phospho 217/221 MEK antibody. The peaks observed are MEK variants which are phosphorylated on the 217 site. Other regulatory and non-regulatory phosphorylations will shift the pI, resulting in a distribution of Raf-phosphorylated MEK species in the graphs above. PD0325901 decreases the presence of phospho 217/221 in SKMEL28 by 30% **but** increases signal in SKBR3 from nearly absent levels in the baseline state.

## Isoform-specific Antibodies Resolve Multiply Phosphorylated Forms of MEK1 and MEK2

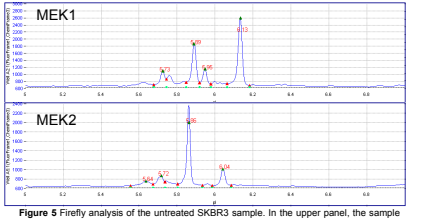


**Figure 5** Firefly analysis of the untreated SKBR3 sample. In the upper panel, the sample is probed with a MEK1 specific antibody, revealing 6 peaks. Phosphatase treatment has shown that all peaks with pI of 5.95 and below are multiply phosphorylated forms of MEK1. All of these variants are modified to species with pI above 6.0 after treatment. By analyzing MEK1 and MEK2 phosphoforms independently, one can analyze phosphorylations even if they reside on conserved regions of the two isoforms.

## Strategy for MEK1 Peak Assignment Using Phospho-specific Antibodies

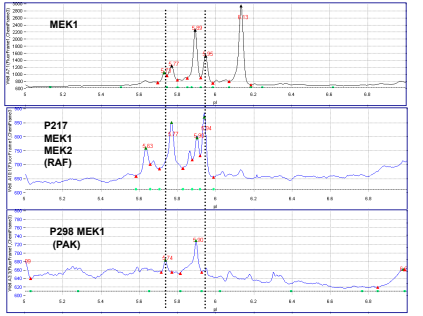


**Figure 6** Quantitative analysis of the MEK1 profile requires assignment of specific phosphorylations to individual peaks. Phospho-specific antibodies are an important tool in peak identification. To assign peaks, we have compared MEK1 variants in the MEK1 profile to MEK1 variants which appear in the phospho-specific antibody profile. For example, the peak at 5.94/5.95 is present in the MEK1 and p217 antibody traces, but not in p298, indicating the peak in the MEK1 profile contains variants with the p217 Raf-specific phosphorylation, but not with the p298 PAK-specific phosphorylation. Likewise, the peak at 5.73/5.74 can represent variants with the PAK-specific phosphorylation but not the Raf-specific one. Further controls using phosphatases, Raf inhibitors and other phospho-specific antibodies will provide insight into the constituents of each peak in the MEK1 antibody profile.

## Conclusions

- Protein identification using Firefly analysis allows for the relative quantitation of variants of a given target protein.
- Firefly identification of ERK variants is feasible, and allows for quantitative determination of phosphorylated protein relative to total protein.
- Firefly analysis of changes in MEK phosphorylation following small molecule MEK inhibition correlates with changes observed by western analysis.
- Further studies are ongoing to complete specific peak identification of MEK1 and MEK2 variants.