Profiling Kinase Phosphorylation using Antibody Arrays

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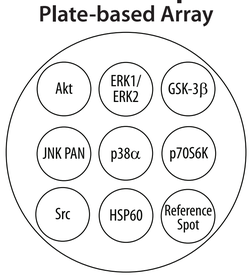
ABSTRACT

Aberrant signaling in the PI 3-Kinase/Akt and Raf/MEK/ERK pathways is associated with the formation of cancerous tumors, while interplay between these two pathways contributes to pharmacological resistance. The goal of this study was to measure the effects of MEK and PI 3-Kinase inhibitors on the phosphorylation of 43 different kinases using antibody arrays as screening tools. In both A549 human lung adenocarcinoma and T47D human ductal breast cancer cells, EGF treatment resulted in increased Akt (S473) and ERK1/2 (T202/Y204,T185/Y187) phosphorylation. In A549 cells, the phosphorylation of Akt was inhibited by the PI 3-Kinase inhibitors AS 605240, LY 294002, and PI 103. ERK phosphorylation in T47D cells was completely inhibited by the MEK inhibitor PD 032590, but displayed resistance to PD 98059 and U0126. Instead, an increase in Akt phosphorylation was observed with U0126 treatment compared to the untreated and other inhibitor treated cells. These results demonstrate the utility of the Human Phospho-Kinase array for monitoring off-target inhibitor responses on interdependent pathways. The dose response of inhibitors on ERK and CREB phosphorylation was measured using arrays and confirmed using ELISA with excellent correlation. By employing a chemiluminescence detection method, no specialized equipment beyond what is typically used to collect Western blot data was required.

MATERIALS & METHODS

Well Map
Plate-based Array

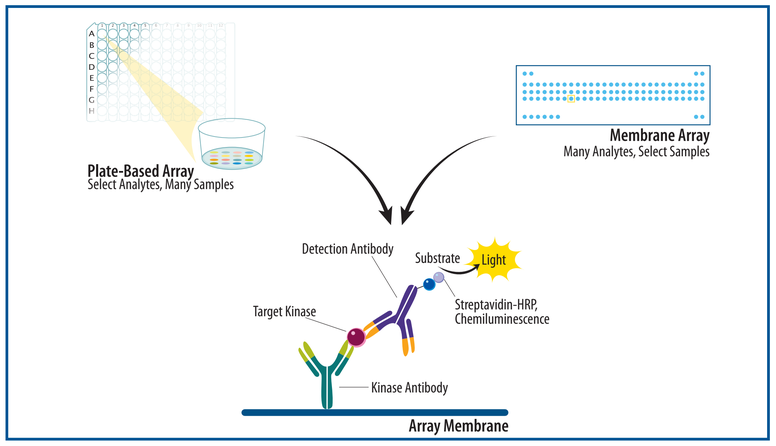


Target	Phosphorylation Site
Akt1	S473
ERK1/ERK2	(T202/Y204, T185/Y187)
GSK-3β	S9
JNK Pan Specific	T183/Y185
p38α	T180/Y182
p70S6K	T421/S424
Src	Y416
HSP60	----

Membrane Content Membrane-based Array		
Akt (S473)	HSP27 (S78/S82)	PRAS40 (T246)
Akt (T308)	HSP60	Pyk2 (Y402)
AMPK α1 (T174)	JNK pan (T183/Y185, T221/Y223)	RSK1/2/3 (S380)
β-catenin	Lck (Y394)	Src (Y419)
Chk-2 (T68)	Lyn (Y397)	STAT2 (Y689)
c-Jun (S63)	MSK1/2 (S376/S360)	STAT3 (S727)
CREB (S133)	p27 (T198)	STAT3 (Y705)
EGF R (Y1068)	p38α (T180/Y182)	STAT5a (Y694)
eNOS (S1177)	p53 (S15)	STAT5a/b (Y694/Y699)
ERK1/2 (T202/Y204, T185/Y187)	p53 (S46)	STAT5b (Y699)
FAK (Y397)	p53 (S392)	STAT6 (Y651)
Fgr (Y412)	p70 S6 Kinase (T421/S424)	TOR (S2448)
Fyn (Y420)	PDGF Rβ (Y751)	WNK-1 (T60)
GSK-3 α/β (S21/S9)	PLC γ1 (Y783)	Yes (Y426)
Hck (Y411)		

Recombinant Human EGF was from R&D Systems (Catalog # 236-EG). Inhibitors were from Tocris, an R&D Systems Company: AS 605240 (Catalog # 3578), LY 294002 (Catalog # 1130), PI 103 (Catalog # 2930), PD 0325901 (Catalog # 4192), PD 98059 (Catalog # 1213), SL 327 (Catalog # 1969), U0126 (Catalog # 1144). Arrays were from R&D Systems: Membrane-based Proteome Profiler™ Phospho-Kinase Array (Catalog # ARY003B), Plate-based Proteome Profiler *96* Phospho-Kinase Array (Catalog # ARZ004). ELISA development kits were from R&D Systems: Phospho-CREB (S133) DuoSet® IC (Catalog # DYC2510), Phospho-ERK1(T202/Y204)/ERK2(T185/Y187) DuoSet IC (Catalog # DYC1018B). Antibodies were from R&D Systems: Anti-Human/Mouse/Rat Phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) Affinity Purified Polyclonal Antibody (Catalog # AF1018), Anti-Human/Mouse/Rat Total ERK1/ERK2 Monoclonal Antibody (Catalog # MAB15761).

ASSAY PRINCIPLE



RESULTS

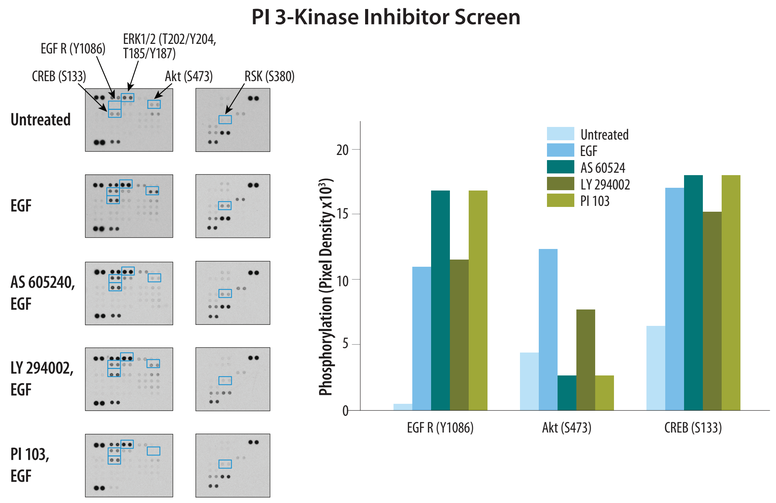


Figure 1. Induction and Inhibition of Kinase Phosphorylation in Lung Adenocarcinoma Cells. The A549 human lung adenocarcinoma cell line was untreated or treated with AS 605240, LY294002, or PI-103 at 5 µM for 3 hours, followed by treatment with 100 ng/mL EGF for 15 minutes. Images of the Proteome Profiler Human Phospho-Kinase membrane array and the corresponding histogram profiles are shown.

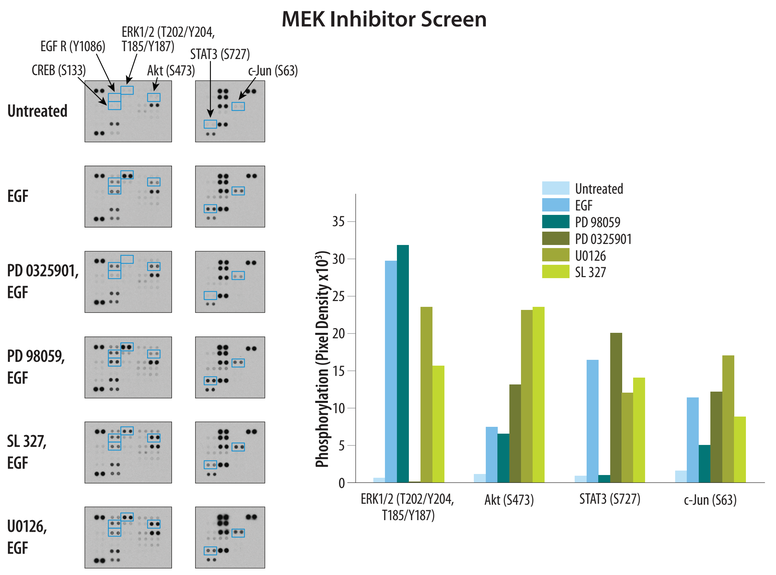


Figure 2. Induction and Inhibition of Kinase Phosphorylation in Breast Cancer Cells. The T47D human ductal breast epithelial cell line was untreated or treated with 10 µM PD 0325901, 20 µM PD 98059, 10 µM SL 327, or 10 µM U0126 for 2 hours, followed by treatment with 100 ng/mL EGF for 15 minutes. Images of Proteome Profiler Human Phospho-Kinase membrane arrays and the corresponding histogram profiles are shown.

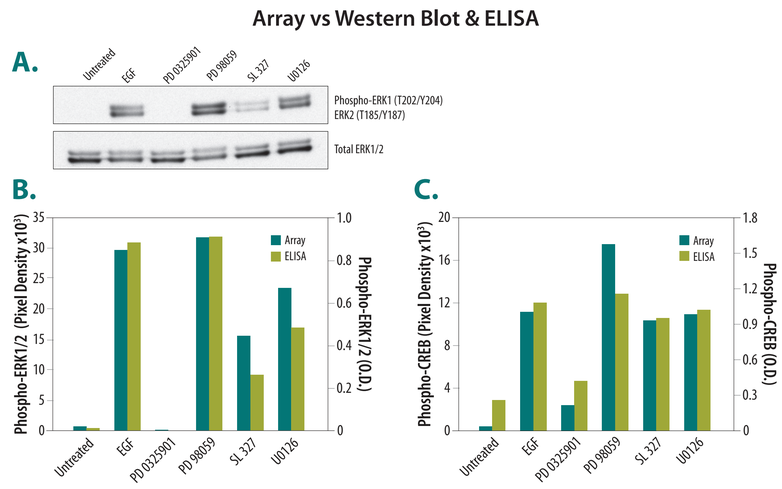


Figure 3. Comparing Array Data to Single Analyte ELISA and Western Blot in a MEK Inhibitor Screen. The T47D human ductal breast epithelial cell line was untreated or treated with 10 µM PD 0325901, 20 µM PD 98059, 10 µM SL 327, or 10 µM U0126 for 2 hours, followed by treatment with 100 ng/mL EGF for 15 minutes. **A.** Phosphorylation was measured using Western blot. Histogram profiles obtained from pixel densities acquired from the Proteome Profiler Array were compared to optical densities obtained from the DuoSet IC ELISA for Phospho-ERK1/2 (T202/Y204,T185/Y187) **(B)** and Phospho-CREB (S133) **(C)**.

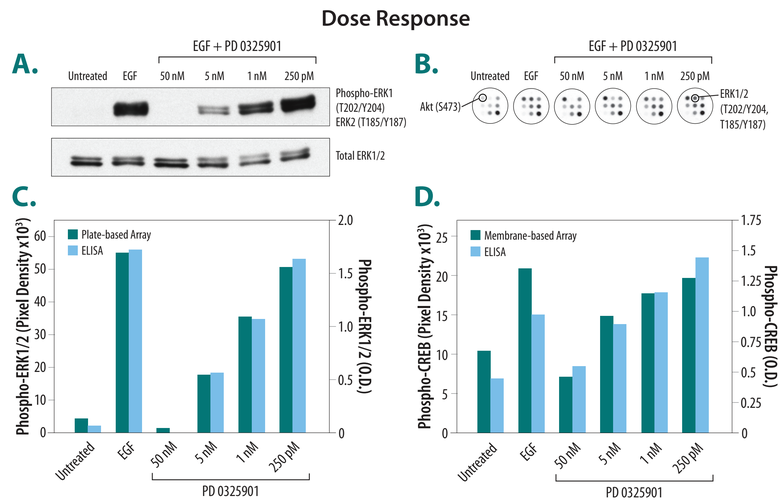


Figure 4. MEK Inhibitor Dose Response Measurements. The T47D human ductal breast epithelial cell line was treated with different concentrations of PD 0325901 for 2 hours, followed by treatment with 100 ng/mL EGF for 5 minutes. **A.** ERK1/2 phosphorylation measured using Western blot. **B.** Images of Proteome Profiler 96 wells. Histogram profiles obtained from pixel densities acquired from the Proteome Profiler 96 Array or the Proteome Profiler Array were compared to optical densities obtained from the DuoSet IC ELISA for Phospho-ERK1/2 (T202/Y204,T185/Y187) **(C)** and Phospho-CREB (S133) **(D)**, respectively.

CONCLUSIONS

The Human Phospho-Kinase array is an economical alternative to traditional methods such as Western blot for screening changes in kinase phosphorylation. Both plate-based and membrane-based arrays required 3.5 hours of hands-on time, making this method far more time-effective than performing multiple Western blots. By employing a chemiluminescence detection method, no specialized equipment beyond what is typically used to collect Western blot data was required. Both arrays are sufficiently sensitive to measure changes in phosphorylation caused by both ligand and inhibitor treatment, and were shown to be comparable to ELISA. This method also allows for the facile evaluation of inhibitor selectivity to off-target kinases.