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APPLICATION NOTE

DETAILED CHARACTERIZATION OF ERK1 AND ERK2 PHOSPHORYLATION

INTRODUCTION

Detecting and quantifying the phosphorylation of extracellular signal-regulated protein kinases ERK1 and ERK2 is criticial to understanding the cell signaling pathways that regulate many basic cellular functions. While both ERK1 and ERK2 have two phosphorylation sites, only doubly phosphorylated isoforms have kinase activity.[1] Western blots are widely used for analyzing protein expression, and phosphorylated protein isoforms may be detected with phospo-specific antibodies. However, traditional Western blots are laborious and require relatively large amounts of sample. Furthermore, ERK1/2 isoforms with different levels of phosphorylation may not be resolved or quantified (see **FIGURE 1**). Thus, Western blots are severly limited in their ability to study ERK1/2 activity.

By contrast, Simple Western Charge is a capillary isoelectric focusing (cIEF) immunoassay that can distinguish and quantify unphosphorylated, mono and dual-phosphorylated isoforms of ERK1/2, allowing for a more accurate determination of ERK activation than Western blots. Using the Simple Western Charge assay, levels of ERK1/2 phosphorylation have been determined in more than 50 different cell and tissue types. This Application Note describes the development of a Simple Western Charge assay to study ERK1/2, which allows resolution and relative quantitation of phospho-ERK isoforms using small quantities of sample. We demonstrate its use in profiling ERK protein expression and activation in breast cancer cell lines.

DEVELOPING THE ERK1/2 ASSAY

Simple Western Charge assays perform protein separations by cIEF. Samples mixed with ampholytes and fluorescent pI standards are loaded into capillaries and focused. After separation, proteins in the capillary are linked to the inner capillary wall by photochemical reaction induced by ultraviolet light exposure. After immobilization, a wash solution, primary antibody, and secondary antibody are passed through the capillary. The secondary antibody is conjugated to horseradish peroxidase (HRP) for chemiluminescent detection.

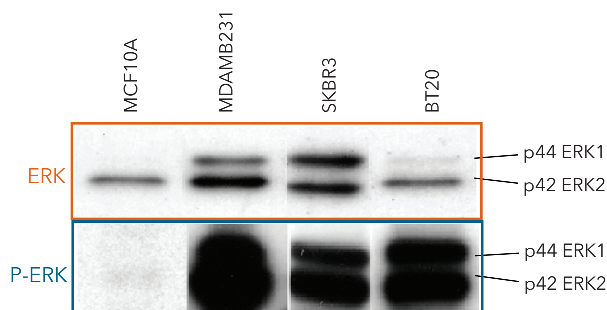

FIGURE 1. Conventional SDS-PAGE Western blot of cell lysates from breast cell line MCF10A and breast cancer cell lines MDAMB231, SKBR3 and BT20, probed with pan-specific ERK antibody (Cell Signaling Technologies, Cat. No. 9102) (top) and phospho-ERK specific antibodies (Cell Signaling Technologies, Cat. No. 9106S and 9101S) (bottom). ERK1 and ERK2 can be distinguished by size and the phospho-isoforms are detectable in the cancer cell lines. However, the Western blot cannot resolve monophospho- and diphosphoisoforms of ERK1 or ERK2 for quantitation.

Detection reagents flow through the capillary and the resulting light signal is detected through the wall of the capillary. A fluorescent image of the pI standards in the capillary is collected, and the pI gradient is defined by the position of the fluorescent pI standards. Aligning the fluorescent signature with the peak profile of the chemiluminescent signal in a capillary allows for the assignment of *pI* values to the probed proteins. By integrating the peaks corresponding to the protein and phospho-protein isoforms and performing a simple calculation, values for relative percentages of phosphorylated protein can be generated for the different isoforms.

HT-29 human colorectal adenocarcinoma cells were used as the assay development model system for ERK peak identification. Cells were treated with insulin plus TNF-alpha for 30 minutes to induce ERK activation. Following activation, cell lysates were immediately prepared from each sample. Multiple capillaries were loaded with the lysates and assayed on the Simple Western Charge system, probing with a series of antibodies specific for unphosphorylated and phosphorylated ERK isoforms. Cell lysate samples were assayed in parallel using Western blots. Western blot and Simple Western Charge data for control (T = 0) and stimulated (T = 30 minutes) samples are shown in **FIGURE 2**.

Conventional SDS-PAGE Western blots were probed with antibodies specific for pan-ERK (ERK1/2), ERK1, ERK2, and phosphorylated ERK1 and ERK2 (pERK1/2). Samples shown are from insulin and TNF-alpha treatment for 0 minutes and 30 minutes. Simple Western Charge analyses of cell lysates were probed with the same antibodies: untreated 0 minutes, 30 minutes insulin, and TNF-alpha treatment. Probing with ERK antibodies with different specificities allowed the peak identifications, as noted.

ERK1/2 PHOSPHORYLATION

The Western blot in **FIGURE 1** shows undetectable levels of phosphorylated ERK1 and ERK2 in the resting normal breast cell line MCF10A, but much higher levels in the breast cancer cell lines MDAMB231, SKBR3, and BT20. Phosphorylation of ERK1 and ERK2 proteins does not increase the molecular weight of the proteins significantly; therefore, monophospho- ERK is not resolvable from diphospho-ERK in a size-based Western blot.

However, phosphorylation does affect the pI of the proteins, making them resolvable and quantifiable in the Simple Western Charge assay. Treatment of MCF10A cells with epidermal growth factor (EGF) will trigger the MAPK signaling pathway and lead to activated ERK1 and ERK2. EGF-treated and untreated MCF10A cell samples were assayed on the Simple Western Charge system with total ERK antibody. Results are shown in **FIGURE 3**. Untreated MCF10A cells showed the presence of unphosphorylated ERK1 and ERK2, but little of the phosphorylated isoforms, similar to the Western blot result. However, upon treatment with EGF, significant levels of monophospho- and diphospho-isoforms of ERK1 and ERK2 were detected, reflecting the activation of ERK activity in these cells.

Breast cancer cell lines expressed different levels of ERK isoforms than the normal breast cell line, as reflected in their Simple Western Charge assay profiles. Simple Western Charge profiles of these cells are shown in **FIGURE 4**. Data derived from peak quantitation of these profiles were plotted, and the relative amount of each isoform of ERK, unphosphorylated and phosphorylated, could be readily compared (see **FIGURES 5 and 6**).

Consistent with the Western blot results, the Simple Western Charge assay detected higher levels of ERK1 and ERK2 phosphorylation in the cancer cell lines than in the normal cells. However, differences could be seen in the relative levels of monophospho- and diphospho-ERK for the different cell lines that were not detected by the Western blot. For example, there were no phosphoisoforms of ERK1 detected in the MCF10A samples, but small amounts of monophospho- and diphospho-ERK2 were detected—about 11 percent relative to the total amount of ERK1/2 protein. The relative amount of diphospho-ERK2 was higher than monophospho-ERK2 for MDAMB231, but the trend was reversed for SKBR3 and BT20. Additional experiments would be required to understand the biological significance of these differences; however, having the ability to discern the relative amounts of monophospho- and diphospho-ERK should allow a more accurate characterization of ERK activation in cell samples.

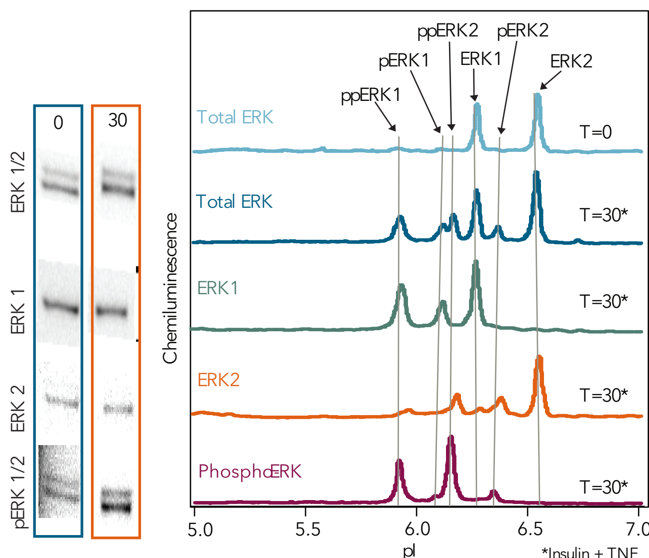

FIGURE 2. Western blot and Simple Western Charge analyses of ERK1 and ERK2 isoforms in cell lysates from HT-29 cells treated with insulin and TNF-alpha.

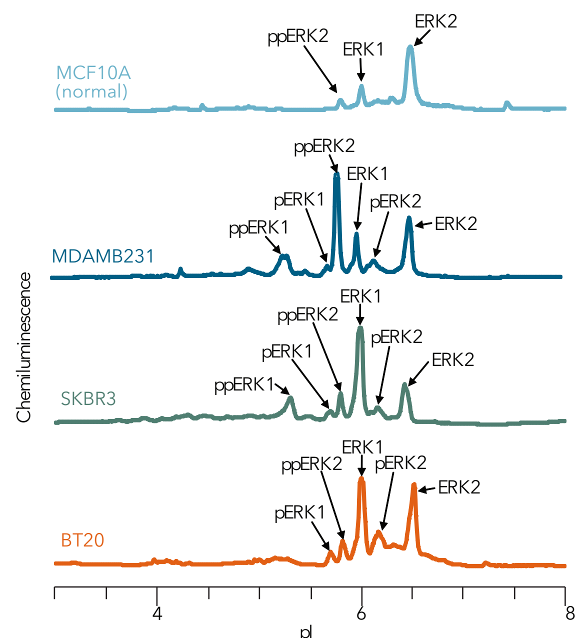

FIGURE 3. Simple Western Charge ERK1/ERK2 assay of MCF10A cells treated with EGF for 0 minutes (untreated, dark blue) and 30 minutes (light blue).

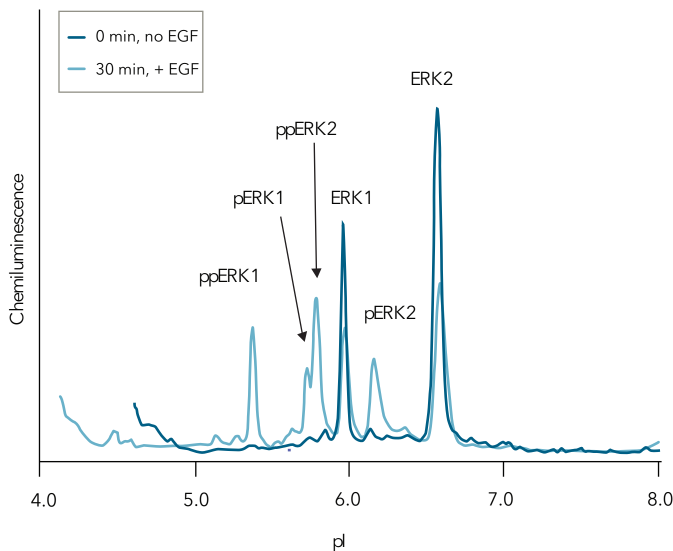

FIGURE 4. Simple Western Charge analysis of ERK1 and ERK2 isoforms in cell lysates from human breast and breast cancer cell lines. Each cell line shows a unique phosphorylation pofile for ERK1/2.

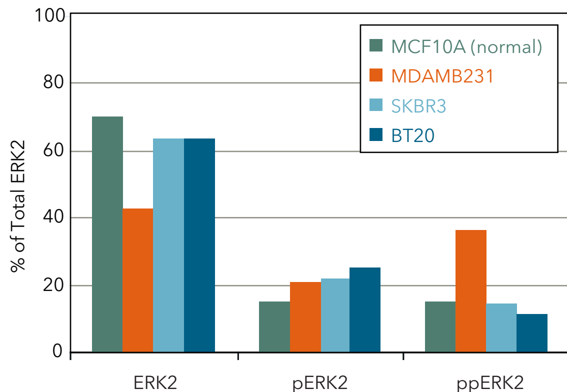

FIGURE 5. Relative levels of unphosphorylated, monophospho- (pERK1) and diphospho-ERK1 (ppERK1) in a normal breast cell line (MCF10A) and three breast cancer cell lines (MDAMB231, SKBR3, BT20). Data derived from quantitation of peaks in Figure 4.

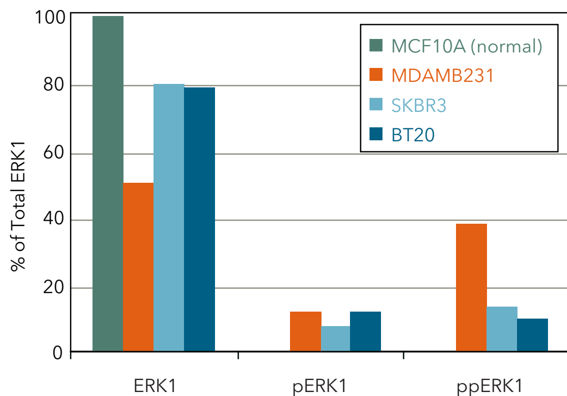

FIGURE 6. Relative levels of unphosphorylated, monophospho- (pERK2) and diphospho-ERK2 (ppERK2) in a normal breast cell line (MCF10A) and three breast cancer cell lines (MDAMB231, SKBR3, BT20). Data derived from quantitation of peaks in Figure 4.

BROAD APPLICABILITY OF SIMPLE WESTERN CHARGE ERK1/2 ASSAY

Simple Western Charge ERK1/2 assays have been run on a wide range of samples. Examples are listed in TABLE 1. The assays are extremely sensitive, capable of measuring protein from as few as 25 cells. The system is fully automated, enabling the researcher to obtain 96 isoform profiles of ERK1/2 in an overnight run.

Drugs or treatments designed to act on ERK activation through interaction with upstream signaling proteins can be screened using the Simple Western Charge assay. The Simple Western Charge ERK1/2 assay can be used to define the functional interactions of different cell signaling proteins in ERK1/2 activation, with respect to both single and dual phosphorylation. Simple Western Charge assays enable researchers to gain insights into the function of cell signaling proteins that cannot be obtained using Western blots.

REFERENCES

1. ERK1/2 MAP kinases: Structure, function, and regulation, R. Roskoski Jr., *Pharmacological Research*, 2012; 66:105-143.

SAMPLE TYPES	SOURCES
Cultured cell lines	Adrenal cancer
Blood (white blood cells)	Bladder cancer
Cerebrospinal fluid	Breast cancer
Tissues	Cervical cancer
Frozen sections	Colorectal cancer
Flow-sorted cells	Kidney cells
Fine needle aspirates	Leukemia
Laser capture microdissected tissue sections	Lung cancer
	Lymphoma
	Pancreatic cells
	Prostate cancer
	Skin cancer and primary melanocytes
	Tumor stem cells
	Xenograft models

TABLE 1. Samples and sources assayed for ERK1/2 phosphorylation using the Simple Western Charge assay.

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