Determination of antibody's specificity towards phosphorylated protein targets with automated in-capillary enzyme treatment and immunoassay

Daryl A Taketa[1], Rainer Grant[2], Tufan Aydogdu[1]

1) ProteinSimple, a Bio-Techne brand, San Jose, CA 2) Bio-Techne, Minneapolis, MN

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ABSTRACT

Protein phosphorylation is a reversible reaction that is integral in numerous signaling cascades. Characterization of signaling cascades has been largely detected by immunoblotting with phospho-specific antibodies, which may or may not have enough specificity or affinity. Currently, a separate lysate without any phosphatase inhibitors or a separate blot is needed to determine an antibody's specificity. Here we describe a simple assay that leverages automation and quantitation with capillary electrophoresis-based immunoassay (CEIA) to assess the specificity of these antibodies with a single lysate preparation. In this study, three lysate models are used: K562 ± TNFα treatment, 50 ng/mL phorbol myristate acetate (PMA) differentiated THP-1 ± 1 µg/mL lipopolysaccharide (LPS) treatment, and cytotoxic T lymphocytes (CTL) ± 10 ng/mL PMA and 500 ng/mL ionomycin treatment. K562 cell lysates are commercially purchased whereas THP-1 lysates are generated in-house. For CTL cells, whole blood cells from a single donor are isolated and expanded with commercially available kits. Expanded CTL cells are then stimulated with PMA and ionomycin for 15 minutes. Untreated and treated lysate samples are separated and captured to the inner lumen of the capillary wall with UV activated crosslink chemistry. Cross-linked proteins are treated with lambda phosphatase for 1 hour followed by the immunoassay to investigate the specificity of antibodies against phosphorylated protein targets respective to each activated pathway using either chemiluminescent or fluorescent detection. Preliminary data suggest phospho-specific signal decreased >90% with no significant changes to the non-specific noise. The method described here eliminates the need for multiple lysate preparations or an additional blot to assess an antibody's specificity to a phosphorylated protein target.

ASSAY PRINCIPLE

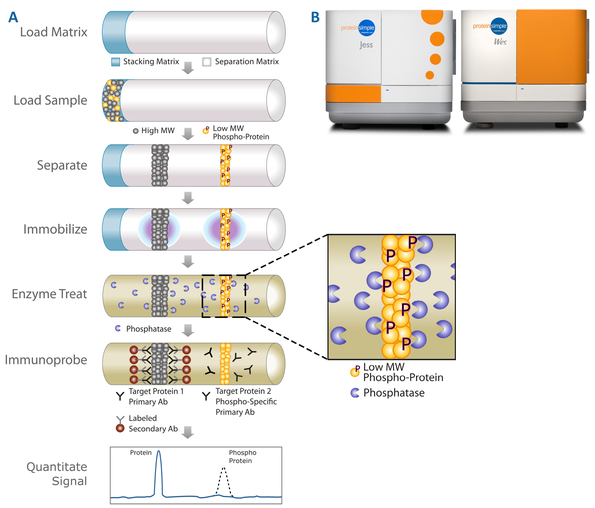

Figure 1. Principle of the automated in-capillary enzyme treatment and immunoassay. (A) Schematic workflow of the automated assay within a self-contained capillary cartridge. Samples are immobilized to the capillary wall via a proprietary UV capture method. Captured proteins are treated with a phosphatase then immunoprobed with an antibody. Target-of-interests are detected with a labeled secondary antibody via HRP chemiluminescent or Near-Infrared (NIR) fluorescent detection. Automation, treatment, and detection of targets can be achieved with either Jess for chemiluminescent and fluorescent detection (B, left) or Wes for chemiluminescent detection only (B, right).

MATERIALS & METHODS

Cytotoxic T Lymphocytes (CTL): EDTA treated whole blood (~250 mL) was obtained from a single donor. $CD8^+$ T cells were negatively selected with Magcellect Human $CD8^+$ T Cell Isolation Kit (R&D Systems #MAGH112). Afterwards, 0.5 x 10^6 cells per mL were expanded using ExCellerate Human T Cell Expansion Media (R&D Systems #CCM030) supplemented with L-glutamine and 25uL of Cloudz™ T Cell Activation Kit – CD3/CD28 (R&D Systems) per mL of total media for 6 days. Following expansion, Cloudz were removed using 1X Release Buffer and placed in a new flask with fresh medium containing no antibody for 24 hours. Cells were then stimulated with 10 ng/mL PMA (Tocris #1201) and 500 ng/mL ionomycin (Tocris #1704) for 15 minutes. Cells were washed then lysed in Lysis Buffer 16 (R&D Systems #895935) supplemented with protease and phosphatase inhibitors.

Differentiated THP-1: THP-1 cells were differentiated into macrophage-like cells with 50 ng/mL PMA in RPMI supplemented with 10% FBS, 1xPen/Strep, and L-Glutamine for 48 to 72 hours. Cells were monitored for differentiation by adherence to a 10 cm plate. Unattached cells were removed by replacing fresh PMA supplemented medium. Cells were then treated with ±1 µg/mL LPS for 24 hours. Cells were washed and then lysed with ProteinSimple's RIPA buffer (#CBS401) supplemented with protease and phosphatase inhibitors.

MCF7: MCF7 cells were grown to 70% confluency in DMEM supplemented with 10% FBS, 1xPen/Strep, and L-Glutamine. Cells were serum starved overnight in complete DMEM medium without any FBS serum. The next day, fresh medium with ±FBS was added with 1 mM Sodium Orthovanadate (New England BioLabs, #P0758S) for 2 hours before cells were washed and then lysed with ProteinSimple's RIPA buffer (#CBS401) supplemented with protease and phosphatase inhibitors.

K562: K562 ± TNFα treatment were commercially purchased from Santa Cruz Biotechnology (#sc-2203 & #sc-24723).

Other Reagents:

Component	Vendor	Catalog #	Dilution
Lambda Phosphatase*	New England BioLabs	P0753S	1:50
Mouse Anti-ERK1/2	Cell Signaling Technology	4696S	1:10
Rabbit Anti-pERK1/2	Cell Signaling Technology	9101S	1:50
Rabbit Anti-p38 MAPK	Cell Signaling Technology	9212S	1:25
Rabbit Anti-p-p38 MAPK	Cell Signaling Technology	9215S	1:25
Mouse Anti-NFκβ p65	Cell Signaling Technology	6956S	1:50
Rabbit Anti-pNFκβ p65	Cell Signaling Technology	3033S	1:50
Rabbit Anti-p70 S6 Kinase	Cell Signaling Technology	9202S	1:10
Mouse Anti-p-p70 S6 Kinase	Cell Signaling Technology	9206S	1:50
Rabbit Anti-Vinculin	Cell Signaling Technology	4650S	1:50
Donkey Anti-Mouse HRP	R&D Systems	HAF018	1:50
Donkey Anti-Rabbit HRP	ThermoFisher Scientific	31458	1:100
Donkey Anti-Mouse AlexaFluor® 647	ThermoFisher Scientific	A-31571	1:100
Donkey Anti-Rabbit AlexaFluor® 647	ThermoFisher Scientific	A-31573	1:100

*Lambda phosphatase was selected for its ability to dephosphorylate serine/threonine as well as tyrosine phosphorylation. Recommended reaction condition is at 30°C for 30 minutes according to manufacturer's guideline but since the temperatures within the automated units are ambient, the reaction time was extended to 1 hour.

Automated In-Capillary Enzyme Treatment And Immunoassays: Assays were developed and conducted on either a Jess or Wes. Default instrument and assay conditions were used except for the following:

- 3-Step Incubation Assay from Compass for Simple Western Version 4.1.0 or above
- Phosphatase Incubation Time = 1 hour
- Primary Antibody Incubation Time = 1 hour
- Secondary Antibody Incubation Time = 30 minutes

RESULTS

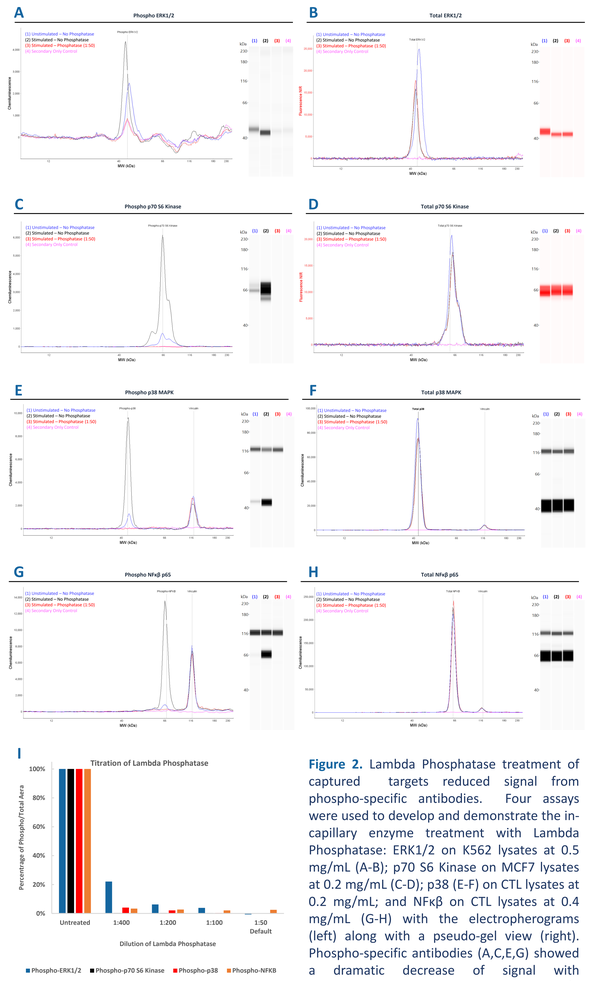

Figure 2. Lambda Phosphatase treatment of captured targets reduced signal from phospho-specific antibodies. Four assays were used to develop and demonstrate the in-capillary enzyme treatment with Lambda Phosphatase: ERK1/2 on K562 lysates at 0.5 mg/mL (A-B); p70 S6 Kinase on MCF7 lysates at 0.2 mg/mL (C-D); p38 (E-F) on CTL lysates at 0.2 mg/mL; and NFκβ on CTL lysates at 0.4 mg/mL (G-H) with the electropherograms (left) along with a pseudo-gel view (right). Phospho-specific antibodies (A,C,E,G) showed a dramatic decrease of signal with phosphatase treatment whereas total protein (B,D,F,H) showed no significant response with in-capillary treatment. (I) Titration of Lambda Phosphatase and the respective quantitation across each assay.

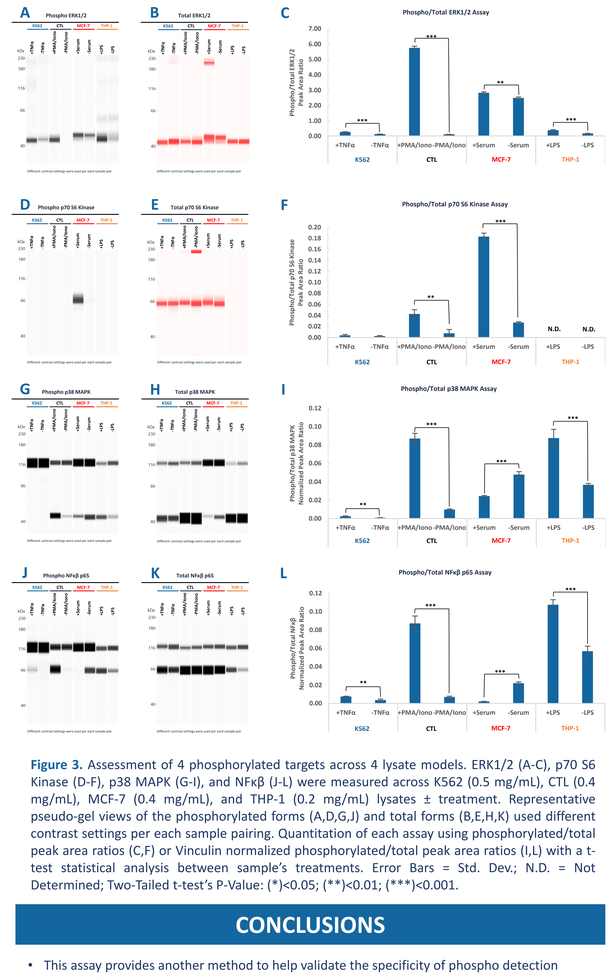

Figure 3. Assessment of 4 phosphorylated targets across 4 lysate models. ERK1/2 (A-C), p70 S6 Kinase (D-F), p38 MAPK (G-I), and NFκβ (J-L) were measured across K562 (0.5 mg/mL), CTL (0.4 mg/mL), MCF-7 (0.2 mg/mL), and THP-1 (0.2 mg/mL) lysates ± treatment. Representative pseudo-gel views of the phosphorylated forms (A,D,G,J) and total forms (B,E,H,K) used different contrast settings per each sample pairing. Quantitation of each assay using phosphorylated/total peak area ratios (C,F) or Vinculin normalized phosphorylated/total peak area ratios (I,L) with a t-test statistical analysis between sample's treatments. Error Bars = Std. Dev.; N.D. = Not Determined; Two-Tailed t-test's P-Value: (*)<0.05; (**)<0.01; (***)<0.001.

CONCLUSIONS

- This assay provides another method to help validate the specificity of phospho detection
- Lambda Phosphatase can reduce phospho-specific signal >90% with no significant impact on other assays
- Provides more confidence on the peak identification and detection with phospho-specific antibodies
- No additional lysates (e.g. without inhibitors) need to be generated
- Automation of the assay is easily achieved with the Simple Western platforms (Jess & Wes)

CONTACT: daryl.taketa@bio-techne.com

bio-techne.com