Novel approach for automated sequential immunoassay for quantitation and characterization of PI3K/AKT pathway proteins

Irina Kazakova, Crystal Tran, Anita Silver and Jessica Dermody ProteinSimple, 3001 Orchard Parkway, San Jose, California 95134

Poster # 6293

INTRODUCTION

The PI3K/Akt signaling pathway modulates cell growth, survival and apoptosis, and this pathway is frequently altered in human cancers, contributing resistance to radiation and chemotherapy treatment. Akt is a target for specific inhibition and recently, a number of small molecules have been developed to improve the pharmacologic properties of known inhibitors like wortmannin and LY294002. However, pan-Akt inhibitors can result in unanticipated side effects due to the lack of specificity for Akt isoforms 1, 2, and 3. Therefore, detection and quantitation of Akt isoforms and their downstream targets for both expression levels and phosphorylation states is crucial for therapeutic drug development. Here we demonstrate application of the RePlex™ to characterize the PI3K/Akt signaling pathway. This approach uses sequential analysis of proteins separated and immobilized in a capillary, by performing either dual immunoassays or immunoassay with total protein on the Simple Western platform using chemiluminescence detection. Assays with control and LY294002 inhibitor-treated samples were developed. Proteins were first separated based on molecular weight via capillary electrophoresis, followed by immobilization via UV-crosslinking. Next, PI3K/Akt pathway targets were sequentially probed in the same capillary with total and phospho-specific antibodies, to determine the phosphorylated fraction relative to the total fraction. Primary antibodies from the first immunoprobe were removed after the detection step with >95% efficiency, as confirmed by re-probing with the same secondary antibody. Target protein loss was negligible due to covalent immobilization to the capillary wall, which was confirmed with re-probing, thus validating the quantitative data generated using this sequential approach. In addition, total protein normalization was performed in tandem with the immunoassay in the same capillary. This approach enables normalization of phosphorylation levels and/or target abundance in cell line or tissue samples, correcting for change in protein content due to treatment, loading, and/or other systematic errors. These results present the utility of the RePlex™ to quickly characterize and quantify proteins involved in signaling pathways targeted during development of cancer therapies.

METHODS

Protocols

- Samples were run on Jess using the 12-230kDa Jess Separation Module (SM-W004), Anti-Mouse Detection Module (DM-002), Anti-Mouse NIR Detection Module (DM-009), Anti-Rabbit Detection Module (DM-001), Total Protein Detection Module (DM-TP01), and RePlex™ Kit (RP-001). Sample, reagent, antibody, and assay plate preparation were performed as described in the Simple Western product insert.
- Samples are automatically loaded into capillaries, separated by size and immobilized to the capillary wall via a proprietary UV capture method. Target proteins are immunoprobed with a primary antibody followed by HRP-labeled secondary antibody for amplified chemiluminescent detection, or NIR-labeled secondary antibody for fluorescent detection.
- The antibodies from the first immunoassay are removed by proprietary RePlex™ reagent, followed by a second immunoassay or Total Protein Assay in the same capillary.
- For the Total Protein Assay, after immobilization proteins are biotinylated and later detected with Streptavidin-HRP.

Materials

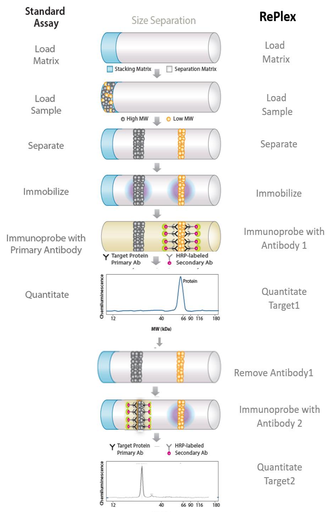
Lysate Samples

- Jurkat Cell Extracts, 1:10 dilution in Sample Buffer (Cell Signaling #9273 AKT Positive control (Jurkat+Calyculin) AKT Non-phosphorylated control (Jurkat+LY294002))
- MCF7 Cell Extracts, 1:10 dilution in Sample Buffer (Cell Signaling #34499 (MCF7 Control untreated, MCF7 hIgF-1 treated)
- Novus Biologicals Human Whole Tissue Lysates: Lung #NB820-59239, Colon # NB820-59205, Kidney # NB820-59231, Brain #NB820-59177, Liver # NB820-59232, Breast# NB820-59203

Primary Antibodies from Cell Signaling Technology with dilution factors

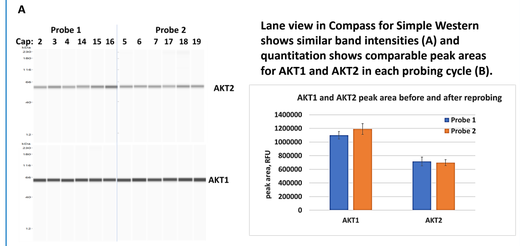
- Mouse Pan AKT Antibody #58295 1:100 for Chemi, 1:50 for NIR
- Rabbit Phospho-AKT(Ser473) Antibody #9271, 1:50
- Rabbit Phospho (Thr308) Antibody #9275 1:50
- Rabbit Phospho-AKT1(Ser473) Antibody #9018 1:50
- Rabbit Phospho-AKT2(Ser473) Antibody #8599 1:50
- Rabbit AKT1 Antibody # 2938 1:100
- Rabbit AKT2 Antibody # 3063 1:100
- Mouse AKT3 Antibody #8018 1:50

Instrument: Simple Western Jess



RESULTS

Figure 1. Signal Intensity and reproducibility for AKT1 and AKT2 in MCF7 lysates, detected in Probe 1 or Probe 2 in a RePlex assay. Regardless of the order in which each protein was probed, AKT1 and AKT2 in MCF7 lysates detected in Probe 1 or Probe 2 in a RePlex assay show excellent reproducibility and similar signal intensity across both probing cycles.



Lane view in Compass for Simple Western shows similar band intensities (A) and quantitation shows comparable peak areas for AKT1 and AKT2 in each probing cycle (B).

Figure 2. Probe 1 antibodies are efficiently removed during a RePlex assay. To determine removal efficiency, primary and secondary antibodies from Probe 1 were removed from the protein in the automated assay and then incubated with the same secondary antibody in Probe 2 to detect any residual primary antibody from Probe 1. **For mouse pan AKT antibody (#58295), greater than 98% of signal from Probe 1 was removed (A). Lane view shows immunoassay signal for Probe 1 and no residual signal for Probe 2 for multiple targets in Jurkat and MCF7 cell lines (B). Removal efficiency (%) for these targets was calculated as (Peak area Probe 1 – Peak area Probe 2)/Peak area Probe 1 x 100 (C).**

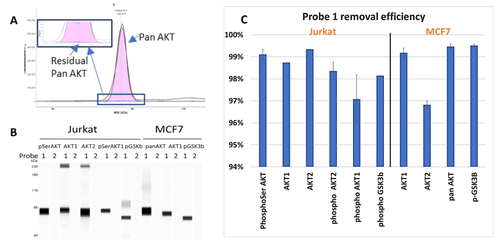
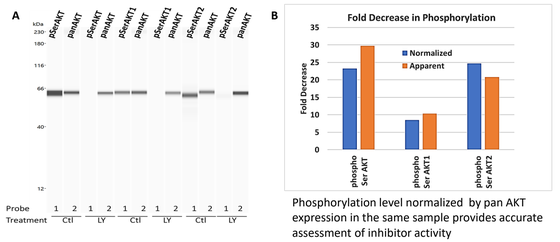
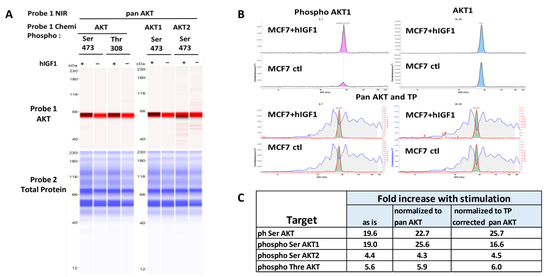


Figure 3: RePlex enables chemiluminescent detection of phospho-specific isoforms of AKT and pan AKT in the same Jurkat lysate sample. **Lane view of pan and isoform-specific AKT phosphorylation and pan AKT signal in control (Ctl) and LY294002-treated (LY) Jurkat lysates (A). Quantitation of peak area shows the raw and normalized fold decrease in phosphorylation in lysates treated with LY294002 inhibitor (B). Phosphorylation was normalized to pan AKT peak area in the same sample.**



Phosphorylation level normalized by pan AKT expression in the same sample provides accurate assessment of inhibitor activity

Figure 4: Automated immunoassay and total protein detection in a single capillary allows for more accurate quantitation of AKT phosphorylation in MCF7 lysates untreated and activated with h-IGF1. **Phospho-AKT or phosphorylated AKT isoforms and pan AKT were detected in Probe 1 using chemiluminescence and NIR fluorescence, respectively, while total protein signal was detected in Probe 2. (A). Example Graph views of AKT1, AKT1 Ser473 phosphorylation, and total protein signal for samples in panel A. Normalization of phosphorylation to pan AKT and total protein signal expression level demonstrates how the normalization method can affect quantitation (C).**



C

Target	Fold increase with stimulation		
	as is	normalized to pan AKT	normalized to TP corrected pan AKT
ph Ser AKT	19.6	22.7	25.7
phospho Ser AKT1	19.0	25.6	16.6
phospho Ser AKT2	4.4	4.3	4.5
phospho Thre AKT	5.6	5.9	6.0

Figure 5. RePlex detection of AKT and concomitant downstream targets for phosphorylation in the same capillary. **Lane view of AKT, GSK3β and cRaf phosphorylation in control (Ctl) and LY294002-treated (LY) Jurkat lysates (A). RePlex enables use of the same secondary antibody in Probe 1 and Probe 2, with no carryover signal, as demonstrated by detection of phosphorylated AKT and GSK3β in sequential probes of the same sample (B). Quantitation shows a decrease in GSK3B and cRaf phosphorylation in LY294002-treated Jurkat lysates (C).**

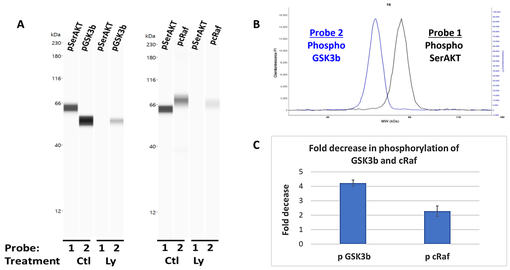
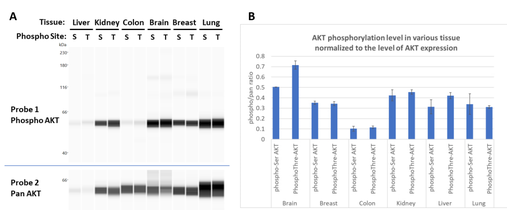


Figure 6: RePlex assays detecting phospho-Ser473 AKT and pan AKT or phospho-Thr308 AKT and pan AKT in the same capillary show variation in phosphorylation status for multiple tissue types and reduces the amount of tissue sample required for multi-target detection. **Lane view (A) and corresponding quantitation of phosphorylation for each tissue sample (B). Phosphorylation signal was normalized to pan AKT signal from the same capillary. (S, Ser473; T, Thr308)**



DISCUSSION

The strip-and-re-probe technique in traditional western is widely used to gain more information from a single blot, thus saving on precious or costly samples. However, this technique is well known to suffer from unpredictable protein loss and typically requires lengthy optimization to achieve optimal stripping efficiency and maximize protein retention on the blot. This makes any quantitative approach using standard strip and re-probe techniques nearly impossible.

The development of the RePlex assay for Simple Western combines the time-saving benefits of an automated immunoassay with multi-target detection and protein normalization in the same capillary, resulting in more data and quantitation with less sample. Optimization of the RePlex approach has shown that signal intensities and reproducibility for the same protein before and after antibody removal were not affected. Therefore, antibody removal using RePlex does not cause protein loss and does not affect epitope binding, which is crucial for quantitation in sequential immunoassays. We attribute this to the proteins being covalently immobilized to the capillary surface in Simple Western assays, unlike protein association with membranes used in western blots, which is driven via hydrophobic interactions.

We have demonstrated here highly effective antibody removal and excellent target protein retention in the sample capillary for multiple targets in the AKT/PI3K pathways using RePlex. Phosphorylation, isoform and pan specific antibodies were used for a subset of targets to assess the overall degree of phosphorylation and evaluate the isoform specific expression levels and phosphorylation states in RePlex assays. Both cell lines as well as tissue lysates were analyzed in Chemiluminescence and NIR Fluorescence modes using RePlex on Simple Western to evaluate multiple targets in the same sample, including phosphorylation level for specific targets relative to either target protein expression or total protein content. This is especially important when differently treated samples must be analyzed independently and compared. Lastly, the highly effective antibody removal in RePlex, even for targets with high signal, allows detection of multiple proteins in one sample using the same antibody species without carryover signal, significantly expanding the antibody pairings used in Probe1 and Probe 2.

CONCLUSION

- **The novel RePlex assay uses the highly sensitive Simple Western quantitative approach to characterize multiple target proteins and perform total protein normalization in the same sample capillary.**
- **Protein integrity and re-probing is not compromised by antibody removal in RePlex assays, providing reliable quantitation and normalization for target protein expression level in multiple samples.**
- **Using RePlex, the AKT signaling pathway was characterized and quantified in the same sample for total and isoform expression levels and phosphorylation states.**
- **Total protein normalization performed in tandem with the immunoassay in the same capillary using RePlex offers a highly sensitive and effective method of normalization.**
- **Performing automated sequential immunoassays using RePlex saves precious samples and provides more data per sample.**

CONTACT: Jessica.Dermody@bio-techne.com
bio-techne.com