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APPLICATION NOTE

# REPROBE YOUR IMMUNOASSAY SAMPLES USING REPLEX WITH JESS


## INTRODUCTION

The identification and quantification of protein expression levels is critical to understanding biological processes like those that drive cancer and other diseases. Unfortunately, limited samples sizes are far too common in these research areas, leaving researchers desiring more data from their limited samples. To measure protein expression by traditional methods like the Western blot, researchers must sacrifice most, if not all, of their sample for only a few data points, which are poorly reproducible and semi-quantitative at best. To overcome these limitations, Simple Western™ with Jess™ is an automated, capillary-based immunoassay that provides highly reproducible and quantitative data with a tiny amount of material. With just 3 µL of sample, Jess offers pg-level sensitivity on up to 25 samples in 3 hours, all in a fully automated fashion. Now, Jess comes with **RePlex™** so that you can reuse the same 3 µL of sample for automated sequential rounds of probing.

## REPLEX WITH SIMPLE WESTERN LETS YOU DO MORE WITH LESS


To gain the most data out of your precious sample, Jess provides a two-step immunoassay that is performed within the same capillary. This new feature, called RePlex removes the antibodies from the first round of probing to perform either a second round of probing with new antibodies or total protein detection. Importantly, RePlex efficiently removes antibodies between probing without compromising the integrity of the immobilized protein or its epitopes, allowing for excellent reproducibility across cycles. With Jess's chemiluminescence and fluorescence channels, you can detect multiple targets per cycle. The second cycle can also be dedicated to total protein detection so that you can normalize your data with confidence. All the steps of RePlex are automatically performed with Jess, providing more data, and lowering the cost of reagents and consumables per result.

## PUTTING REPLEX TO THE TEST

In this Application Note, we show how you can use RePlex to detect and quantify pan and phospho isoforms of proteins such as those in the PI3K/Akt signaling pathway. This pathway modulates cell growth, survival, and apoptosis, and it is frequently altered in human cancers, contributing resistance to radiation and chemotherapy treatment[1,2]. First, we provide evidence that RePlex efficiently removes antibodies between probing without compromising the integrity of the immobilized protein or its epitopes. Then we use RePlex to multiplex antibodies to phosphorylated isoforms and pan isoforms of Akt in the first cycle, followed by a second cycle to normalize the data by total protein detection. Finally, we extend RePlex to characterize concomitant downstream targets and protein expression in different tissue types.

## MATERIALS AND METHODS

Experiments were performed with the products listed in **Table 1** and antibodies listed in **Table 2**.

**TABLE 1**

| PRODUCT | VENDOR | PRODUCT NUMBER |
|---|---|---|
| 12-230 kDa Jess/Wes Separation Module | ProteinSimple | SM-W004 |
| Anti-Rabbit Detection Module | ProteinSimple | DM-001 |
| Anti-Mouse Detection Module | ProteinSimple | DM-002 |
| Anti-Mouse NIR Detection Module | ProteinSimple | DM-009 |
| Total Protein Detection Module | ProteinSimple | DM-TP01 |
| RePlex Kit | ProteinSimple | RP-001 |
| Jurkat Cell Extract | Cell Signaling Technologies | 9273 |
| MCF7 Cell Extract | Cell Signaling Technologies | 34499 |
| Lung Human Whole Tissue Lysate | Novus Biologicals | NB820-59239 |
| Colon Human Whole Tissue Lysate | Novus Biologicals | NB820-59205 |
| Kidney Human Whole Tissue Lysate | Novus Biologicals | NB820-59231 |
| Brain Human Whole Tissue Lysate | Novus Biologicals | NB820-59177 |
| Liver Human Whole Tissue Lysate | Novus Biologicals | NB820-59232 |
| Breast Human Whole Tissue Lysate | Novus Biologicals | NB820-59203 |

**TABLE 1. Products used in this study.**

**TABLE 2**

| ANTIBODY | VENDOR | PRODUCT NUMBER | DILUTION FACTOR |
|---|---|---|---|
| Mouse Pan AKT | Cell Signaling Technologies | 58295 | 1:100 (Chemi)<br>1:50 (NIR) |
| Rabbit Phospho-AKT(Ser473) | Cell Signaling Technologies | 9271 | 1:50 |
| Rabbit Phospho (Thr308) | Cell Signaling Technologies | 9275 | 1:50 |
| Rabbit Phospho-AKT1(Ser473) | Cell Signaling Technologies | 9018 | 1:50 |
| Rabbit Phospho-AKT2(Ser473) | Cell Signaling Technologies | 8599 | 1:50 |
| Rabbit AKT1 | Cell Signaling Technologies | 2938 | 1:100 |
| Rabbit AKT2 | Cell Signaling Technologies | 3063 | 1:100 |
| Mouse AKT3 Antibody | Cell Signaling Technologies | 8018 | 1:50 |

**TABLE 2. Antibodies used in this study.** Dilution factors used in the study are listed.

Sample, reagent, antibody, and assay plate preparation were performed as described in the Simple Western product inserts. Lysates were diluted 1:10 in Sample Buffer prior to mixing with fluorescent standards. Target proteins were 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 were removed using the RePlex reagent, followed by a second immunoassay or Total Protein Assay in the same capillary. For the Total Protein Assay, immobilized proteins were biotinylated and later detected with Streptavidin-HRP.

## PROTEIN DETECTION IS UNAFFECTED BETWEEN REPLEX CYCLES

RePlex™ with Jess™ enables two-step immunoassays that rely on the removal of antibodies from the first round of probing (Probe 1) prior to a second round of probing with fresh antibodies (Probe 2). To demonstrate that signal intensity and reproducibility are not compromised between Probe 1 and Probe 2, we measured the signal intensity of AKT1 and AKT2 antibodies in MCF7 lysate in the two probing steps. Lane view showed similar band intensities (**Figure 1A**), while quantitation of AKT1 and AKT2 confirmed that peak areas were very similar for Probe 1 and Probe 2 when looking at each protein target (**Figure 1B**). 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 showed excellent reproducibility and similar signal intensity across both probing cycles. This gives us confidence that protein integrity and re-probing are not compromised by antibody removal in RePlex assays.

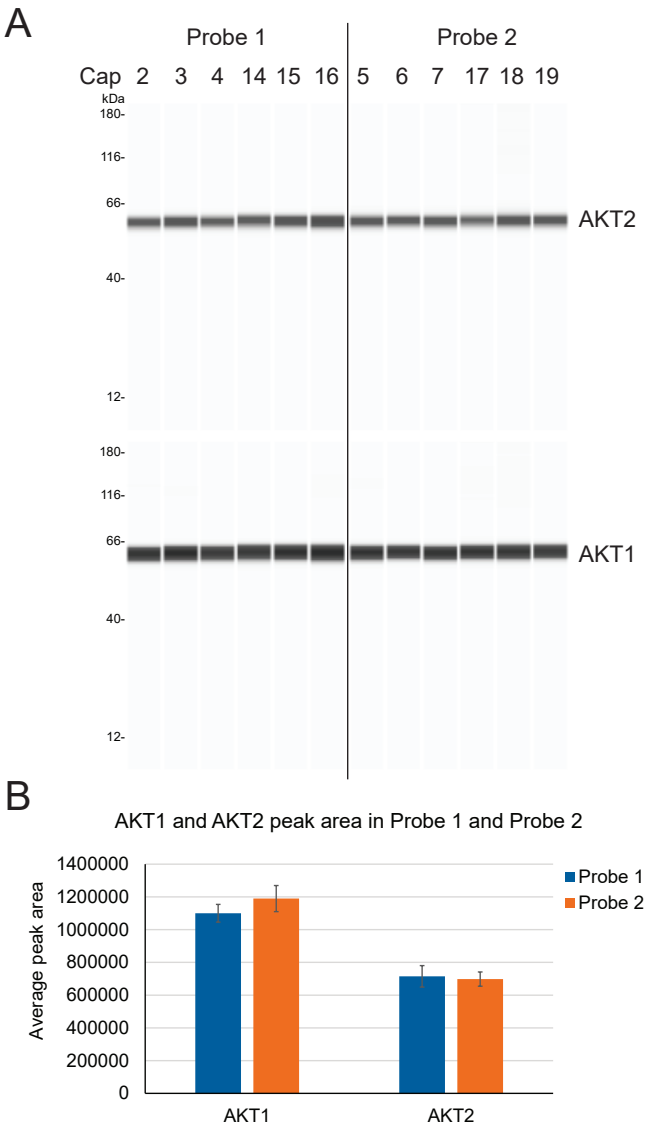


**FIGURE 1. Signal intensity and reproducibility for AKT1 and AKT2 in MCF7 lysates, detected in Probe 1 or Probe 2 in a RePlex assay.** (A) Lane view in Compass for Simple Western shows similar band intensities, and (B) quantitation shows comparable peak areas for AKT1 and AKT2 in each probing cycle. Error bars represent standard deviations of the means.

## ANTIBODIES ARE EFFICIENTLY REMOVED WHEN USING REPLEX™ WITH JESS™

A critical step in RePlex is the efficient removal of antibodies after the first round of probing. Thus, we assessed removal efficiency of antibodies against pan and phosphorylated AKT on Jurkat and MCF7 lysates. To do so, the primary and secondary antibodies from Probe 1 were removed in the automated assay. The same secondary antibody that was used in Probe 1 was then used in Probe 2 without the addition of new primary antibody to detect any residual primary antibody from Probe 1. The removal efficiency was measured as the percent of signal that was detected in Probe 2 versus the original signal in Probe 1. From this analysis, we found that greater than 98% of signal was removed for pan AKT (**Figure 2A**). The lane view and removal efficiency for multiple AKT antibodies are shown in **Figures 2B** and **2C**, respectively. All antibodies tested showed greater than 96% removal efficiency, with most of the antibodies tested showing 98% or higher. These data provide evidence that a second round of antibody probing may be performed with little to no crossover from the first round of probing.

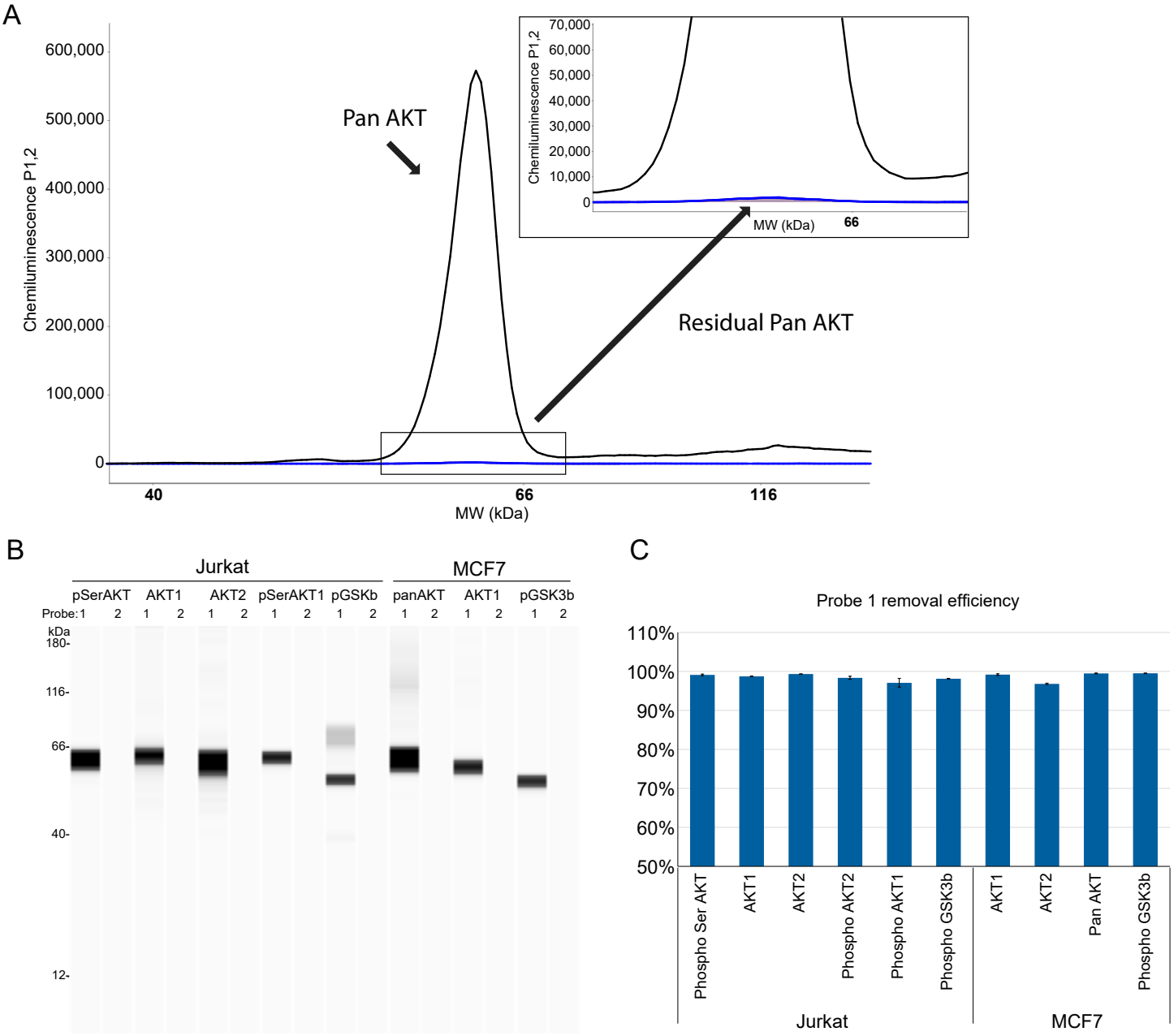


**FIGURE 2. First antibody probes are efficiently removed during the RePlex assay.** (A) More than 98% of the signal from Probe 1 was removed for mouse pan AKT antibody. (B) Lane view shows the immunoassay signal for Probe 1 and no residual signal for Probe 2 for multiple targets in Jurkat and MCF7 cell lines. (C) Removal efficiencies (%) for these targets were calculated as (Peak area Probe 1 – Peak area Probe 2)/Peak area Probe 1 x 100. Error bars represent standard deviations of the means.

## IMMUNOASSAY AND TOTAL PROTEIN DETECTION SEAMLESSLY BACK-TO-BACK

RePlex™ with Jess™ performs sequential immunoassays seamlessly back-to-back. In a similar manner, it can also be used to perform an immunoassay in the first round and total protein detection in the same capillary. To demonstrate this, we used MCF7 cells untreated and treated with human IGF1 (hIGF1). We were able to detect pan and phosphorylated AKT in Probe 1 by multiplexing the primary and secondary antibodies and then subsequently detect total protein signal in Probe 2 after removal of the antibodies. The lane view in **Figure 3A** shows phosphorylated AKT proteins detected using chemiluminescence, as indicated by black bands, and pan AKT using NIR detection, as indicated by the red bands in Probe 1. Total protein detection from Probe 2 is shown in blue. **Figure 3B** shows representative electropherograms of the signal for the various AKT antibodies and total protein signal. Automated normalization of phosphorylated and pan AKT signal to total protein signal is displayed in the Peaks Table in Compass for Simple Western (**Table 3**).

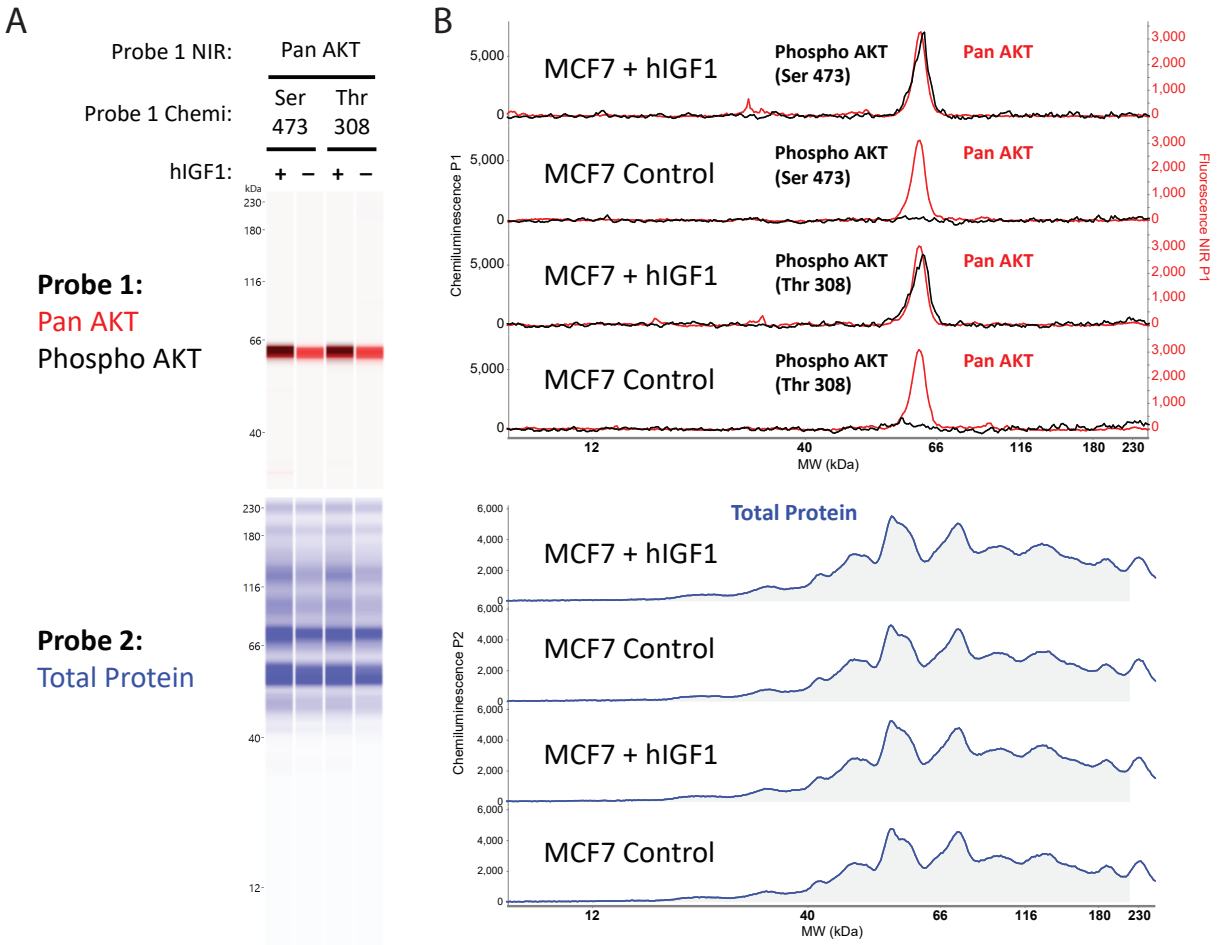


FIGURE 3. Automated immunoassay and total protein detection in a single capillary of AKT phosphorylation in MCF7 lysates treated and untreated with hIGF1. (A) Phosphorylated AKT (phospho AKT) isoforms and pan AKT were detected in Probe 1 using chemiluminescence and NIR fluorescence, respectively, while total protein signal was detected in Probe 2. (B) Example graph views of phospho AKT and pan AKT (top), and total protein signal (bottom) for samples in panel A.

Peaks | Capillaries

| Sample | Primary | Cap | Name | MW (kDa) | Area | Corr. Area | Channel |
|---|---|---|---|---|---|---|---|
| MCF7+hIGF1 | r p-SerAKT/m pan AKT | P1:2 | phospho Ser AKT | 63 | 69343.5 | 69343.5 | CHEMI |
| MCF7 ctl | r p-SerAKT/m pan AKT | P1:3 | phospho Ser AKT | 60 | 3132.6 | 3537.3 | CHEMI |
| MCF7+hIGF1 | r p-Thr AKT/m pan AKT | P1:4 | phospho Thr AKT | 63 | 68367.4 | 70957.8 | CHEMI |
| MCF7 ctl | r p-Thr AKT/m pan AKT | P1:5 | phospho Thr AKT | 59 | 10945.2 | 12912.8 | CHEMI |
| MCF7+hIGF1 | r p-SerAKT/m pan AKT | P1:2 | pan AKT | 63 | 30187.7 | 30187.7 | NIR |
| MCF7 ctl | r p-SerAKT/m pan AKT | P1:3 | pan AKT | 62 | 30678.9 | 34642.1 | NIR |
| MCF7+hIGF1 | r p-Thr AKT/m pan AKT | P1:4 | pan AKT | 63 | 30051.6 | 31190.2 | NIR |
| MCF7 ctl | r p-Thr AKT/m pan AKT | P1:5 | pan AKT | 62 | 32878.5 | 38789.0 | NIR |

TABLE 3. Peaks Table in Compass for Simple Western shows automated normalization of phosphorylated and pan AKT signal to total protein signal demonstrates quantitation of target protein expression.

## MULTI-TARGET DETECTON USING REPLEX™

In addition to probing the same target with different antibodies, we also used RePlex to characterize other signaling proteins along with AKT detection, including cRaf and GSK3 beta (GSK3b). Using lysates from control and LY294002-treated Jurkat cells, we could clearly detect changes in phosphorylation status for multiple proteins in the same sample, as shown in Panel A and quantified in Panel C in **Figure 4**. Furthermore, due to the high primary antibody removal efficiency of RePlex, the same secondary antibody species could be used in both Probe 1 and Probe 2, without any carryover signal from Probe 1. This is seen in the electropherograms in Panel B where only a single peak is detected for phosphorylated GSK3b in Probe 2, the blue line, and no residual peak is present from the phosphorylated AKT antibody that was used in Probe 1 even though both primary antibodies are rabbit.

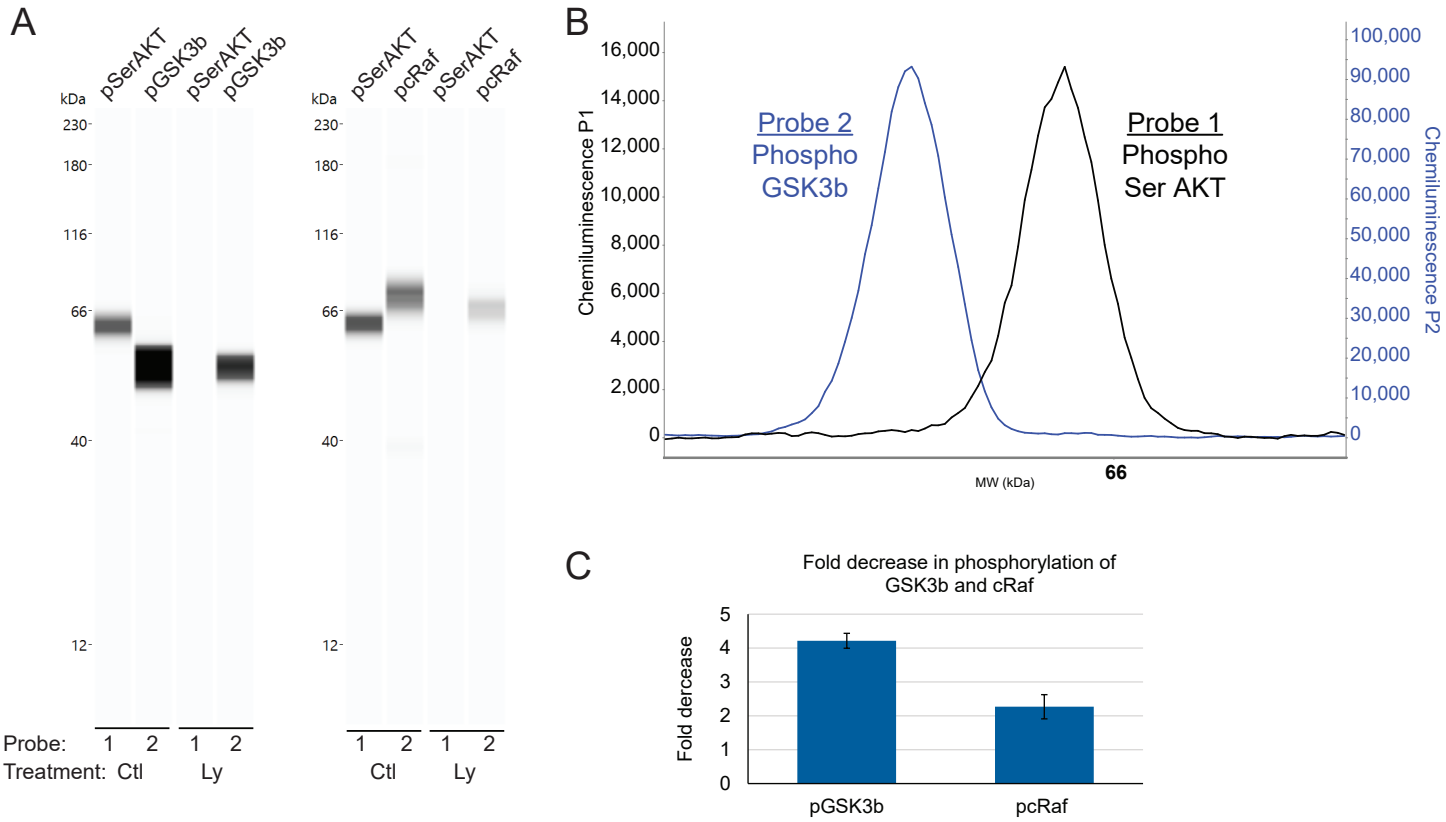


**FIGURE 4. RePlex detection of concomitant phosphorylation for AKT and downstream targets in the same capillary.** (A) Lane view of AKT, GSK3b and cRaf phosphorylation in control (Ctl) and LY294002-treated (LY) Jurkat lysates. (B) RePlex enables use of the same secondary antibody in Probe 1 and Probe 2, with no carryover signal, as demonstrated by detection of phosphorylated AKT and GSK3b using rabbit-derived primary antibodies in sequential probes of the same sample. (C) Quantitation shows a decrease in GSK3b and cRaf phosphorylation in LY294002-treated Jurkat lysates. Error bars represent standard deviations of the means.

As final demonstration of the utility of RePlex™, we characterized phosphorylation status of AKT in multiple tissue types. **Figure 5** includes detection of pan and serine or threonine phosphorylated AKT in the same capillary for multiple tissue types, including liver, kidney, colon, brain, breast, and lung. Lane view shows Probe 1 and Probe 2 for each sample (**Figure 5A**), while the graph shows phosphorylation signal normalized to pan AKT signal (**Figure 5B**). As might be expected, different tissues showed both different levels of AKT expression and phosphorylation.

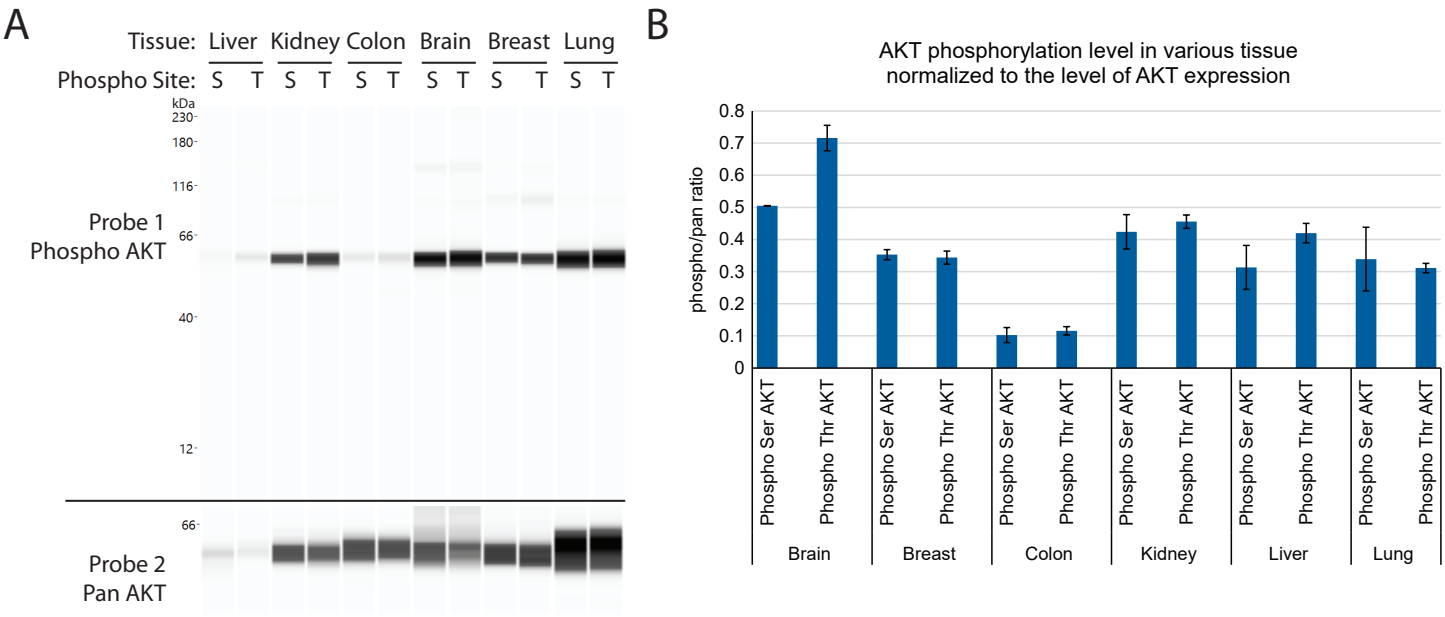


**FIGURE 5. RePlex assays detecting phospho-Ser473 AKT and pan AKT or phospho-Thr308 AKT and pan AKT in the same capillary show variation in phosphorylation status for multiple tissue types and reduces the amount of tissue sample required for multi-target detection.** (A) Lane view and (B) corresponding quantitation of phosphorylation for each tissue sample. Phosphorylation signal was normalized to pan AKT signal from the same capillary. Error bars represent standard deviations of the means. (S, Ser473; T, Thr308)

## REPLEX IS THE FUTURE OF SEQUENTIAL IMMUNOASSAYS

The strip-and-reprobe technique in traditional Western blot 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 reprobe techniques nearly impossible.

By contrast, the RePlex™ assay developed for the Simple Western™ platform Jess™ is an optimized and automated two-step immunoassay that enables even more data to be generated per 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 Probe 1 and Probe 2.

In summary, 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.

## REFERENCES


1. Akt regulates cell survival and apoptosis at a postmitochondrial level, H Zhou, XM Li, J Meinkoth and RN Pittman, *Journal of Cell Biology*, 2000; 151(3):483-94.

2. The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change, H Yamaguchi and HG Wang, *Oncogene*, 2001; 20:7779-86.



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