Analyzing Phospho-Akt from Cell Culture & Tumor Lysates Using BioScale's Acoustic Membrane and Microparticle (AMMP™) technology on the ViBE™ BioAnalyzer

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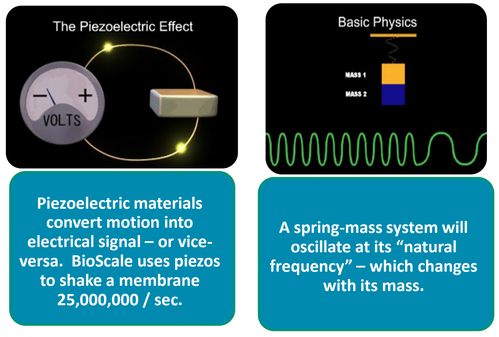
Abstract: The protein kinase AKT (1), also known as protein kinase B (PKB) or Rac, is one of the best characterized targets within the phosphoinositide 3-kinase (PI3K) pathway. The PI3Ks generate phosphoinositol lipids implicated in the regulation of cell growth, proliferation, survival, differentiation, and cytoskeletal rearrangement. Akt is activated through recruitment to cellular membranes by PI3K lipid products and by subsequent phosphorylation at Thr308 by PDK1 followed by phosphorylation within the carboxy terminus at Ser473 by mTOR (2). Activation of Akt promotes cell survival by inhibiting apoptosis through phosphorylation of several downstream targets. Thus, blocking the PI3K signaling pathway has potential implications towards the development of anti-cancer therapeutics.

We sought to develop a strategy for monitoring the inhibition of pSer473-Akt using a novel microfluidic frequency modulating technology from BioScale called AMMP™ using both tissue culture and mouse xenografts. Toward that goal, we selected a pair of commercially available monoclonal antibodies against both pSer473-Akt and pan-Akt. These antibodies were each affinity tagged with either biotin or fluorescein. Using the ViBE™ BioAnalyzer a standard curve was generated with in vitro phosphorylated (p473) recombinant Akt2. Using that derived standard curve we then compared samples treated or untreated with PI3K inhibitors from either cell culture SKOV3 and WM266.4 cells or xenograft tumor lysates using the ViBE™ BioAnalyzer. Our results show clearly that the BioScale technology compares favorably with data obtained from Li-Cor Western Blot analysis and, most importantly, is far less labor intensive than the Western Blot procedure.

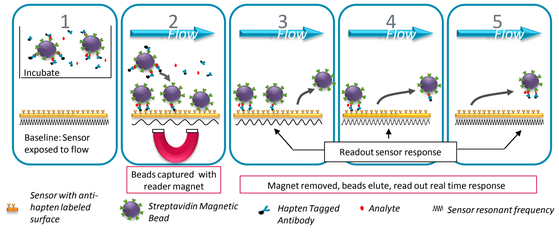
References:

1. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Franke, T. F.; Yang, S.-I.; Chan, T. O.; Datta, K.; Kaziauskas, A.; Morrison, D. K.; Kaplan, D. R.; Tsichlis, P. N., *Cell* 1995 81: 727-736.
2. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B. *Cell.* 2006 Oct 6;127(1):125-37.

AMMP™ Technology Overview

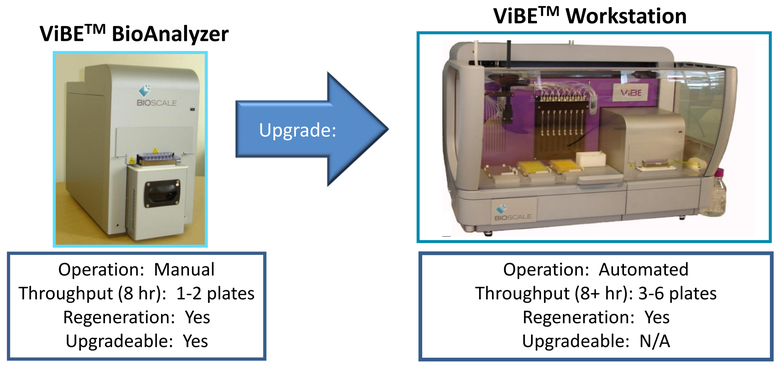


Traditional assay format with novel detection technology allows better results, easier use and faster assay development



1. **Sample is first incubated with streptavidin magnetic capture beads and complementary antibodies each tagged with either a hapten or biotin.**
2. **Beads are collected on the biosensor surface by the applying a magnetic field.**
3. **Beads are briefly washed with buffer while still engaged by the magnet to ensure capture by the anti-hapten antibodies at the membrane surface.**
4. **Magnetic field is released and beads without capture analyte are washed away.**
5. **Under continuing flow, beads elute according to their analyte load.**

Instrumentation:



Results:

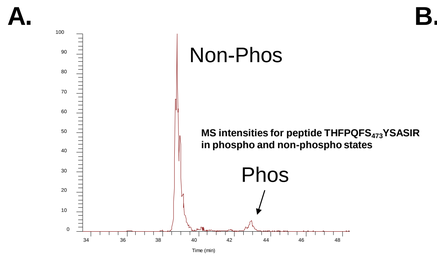
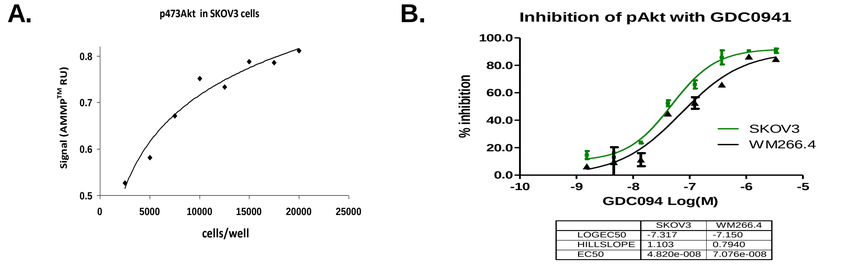


Fig 1. Establishing standard curve of pSer473-Akt with ViBE™ BioAnalyzer (Panel B) using in vitro phosphorylated recombinant GST-Akt (Panel A). Recombinant GST-tagged-Akt2 was phosphorylated in vitro with mTORC2 complex. The percentage of phosphorylation was analyzed using mass spectrometry (Panel A). This batch of pSer473-Akt was used to establish a standard curve (Panel B) titrated over an eight point three-fold dilution series in buffers recommended by the manufacturers. Complementary pairs of anti-Akt and anti-pSer473-Akt antibodies (Cell Signaling) were labeled with either biotin or fluorescein for use with the ViBE™ BioAnalyzer as directed by the manufacturer.



	SKOV3	WM266.4
LOGEC50	-7.317	-7.150
HILLSLOPE	1.103	0.7940
EC50	4.820e-008	7.076e-008

Fig 2. Titration of cell culture density (Panel A) and compound inhibition of pSer473-Akt (Panel B) as analyzed by ViBE™ BioAnalyzer. SKOV3 cells were cultured in dilution series of cell density for 24 hrs prior to in situ lysis with 20 ul mPER buffer (Pierce) in 96 well culture plate. Capture beads and tagged antibody of the same pair as described in Fig. 1 were directly incubated in culture plate before loading to the BioAnalyzer. Cell density for drug IC_{50} assay can be determined based on this data (Panel A). IC_{50}s of pSer473-Akt by GDC0941 were analyzed on SKOV3 or WM266.4 cells at 15,000 cells/well in 96 well plate (Panel B). IC_{50}s are consistent with data using other assay methods (not shown). Results shown are representative of duplicate experiments with standard errors of less than 10%.

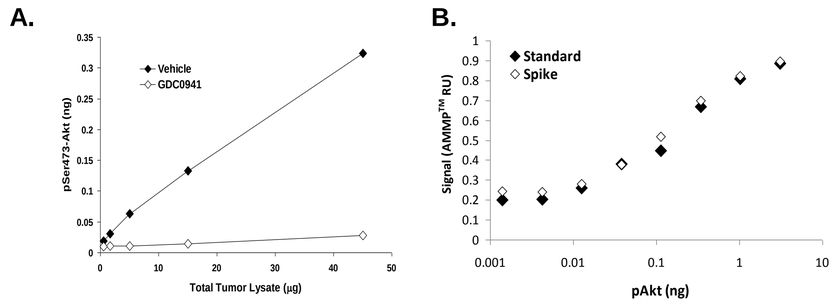


Fig 3. Titration of WM266.4 xenografts tumor lysate for inhibition of pSer473-Akt with either a vehicle or 200mg/kg GDC0941 treatment as analyzed by ViBE™ BioAnalyzer (Panel A) and recovery of spiked recombinant pSer473-Akt in GDC0941 treated tumor lysate (Panel B). Tumor bearing mice were treated PO with either a vehicle or 200 mg/kg GDC0941 compound and WM266.4 samples excised following 2 hrs dose. Tumor tissues were lysed in mPER buffer. Protein concentrations were determined by BCA and normalized for sample addition for each assay. For titration assay (panel A), AMMP™ signal was normalized to the pSer473-Akt signal from Fig.1 using their commercial software package. Phosphorylated recombinant Akt were analyzed as standard curve alone or standard series doses spiked into 5 µg GDC0941 treated tumor lysate, which contains background pAkt. Data indicates 100% recovery of spiking standard (panel B). Results shown are representative of at least duplicate experiments with standard errors of less than 10%.

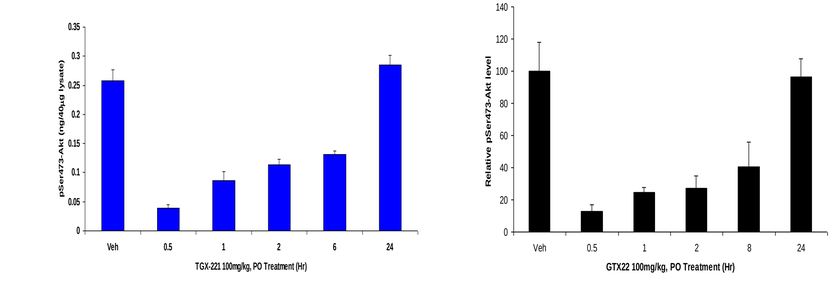


Fig 4. Inhibition of pSer473-Akt from WM266.4 xenogratfts over a time course of a single dose of 100 mg/kg GTX22 treatment as analyzed by either ViBE™ BioAnalyzer (Panel A) or Western Li-Cor (Panel B). Tumor bearing mice were treated PO with 100 mg/kg GTX22 compound and WM266.4 samples excised at each designated time point. Protein concentrations were determined by BCA for either vehicle or drug treated sample. Using the same complementary pairs of Akt/pAkt antibodies as described in Fig. 1 for the ViBE™ assay (panel A) and anti-pSer473-Akt antibody by Western blot Li-Cor analysis (panel B). In the case of the ViBE™ assay the AMMP™ signal was normalized to the pSer473-Akt signal from Fig.1 using their commercial software package. Results shown are representative of at least duplicate experiments with standard errors of less than 10%.

Conclusions:

1. Samples derived from a variety of sources can be readily analyzed using the ViBE™ BioAnalyzer producing the same quality results from recombinant, tissue culture or *in vivo* tumor xenografts.
2. Unlike Western blot Li-Cor the ViBE™ BioAnalyzer affords rapid reproducible and quantitative analysis of all sample types.