Comparative Analysis of *In Vivo* and *In Vitro* Expression of Kinases in the MAPK and PI3K Pathways

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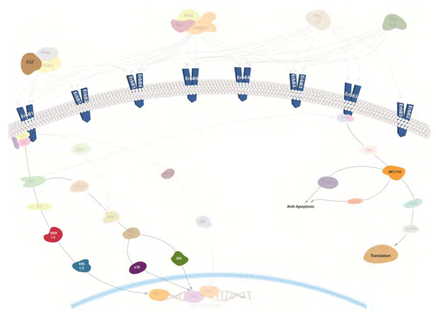
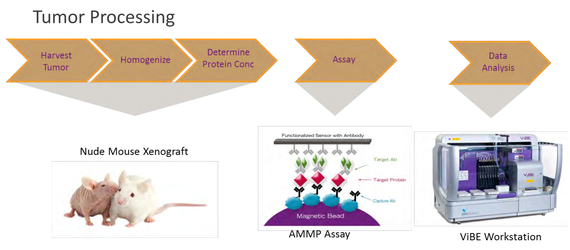
Abstract:

The *mitogen-activated protein kinase* (MAPK) and *phosphoinositide 3-kinase* (PI3K-AKT) cascades are signal transduction pathways which heavily influence the regulation of cell proliferation, differentiation, and survival, and as such, are implicated in a wide variety of cancers. The ability to measure the activation states and molecular interactions of the kinases comprising these pathways are crucial in the efficient and effective development of anti-oncological pharmaceuticals and therapeutics. Here we describe the utility of non-optical acoustic membrane microparticle (AMMP®) technology to assess and quantify the activation state of several implicated kinases: EGFR, p38, and AKT. Lysates were prepared from several human tumor cell lines, with or without stimulation of surface ligand receptors or mitogen stimulation and compared with lysates prepared from xenografted tumors derived from the same cell lines. The correlation of measurement of kinases and receptors in tumor cell lines with the same cell line xenografts was excellent. The number of cells contained in specific amounts of cell lysate has been determined, allowing the elucidation of the amounts of these analytes in samples representing very low cell counts. Consequently, the use of AMMP technology has permitted measuring the activation states of multiple kinases, from fewer than 2000 cultured or xenografted tumor cells per assessment. Herein we also describe a quantitation method by which phospho- and Total-AKT can be measured in ng/mL quantities and provide correlation data by which we have been able to assess quantitation feasibility.

MAP Kinase & PI3K Pathway



From Mouse to AMMP® Assay to ViBE® Analysis



Materials and Methods

Sample Preparation for the Detection of Target Analytes in Cell Line Lysates

Human epidermoid carcinoma cells A431 (ATCC#CRL-1555) were grown to approximately 80% confluence in T75 flasks according to standard cell culture techniques. A431 cells were stimulated for EGFR production with 100 µg recombinant epidermal growth factor (R&D Systems) in serum free medium for 10 minutes. Negative control cells were incubated with serum free medium without stimulant, also for 10 minutes.

Following treatment, the A431 medium was aspirated and replaced with 3 mL TPER (Tissue Protein Extraction Reagent, Pierce) to lift the cells from the plastic flasks. Additional scraping was required to release the cells from the plastic. The resulting cell suspension was incubated for approximately 15 minutes on ice with vortexing every five minutes for effective cell lysis.

HEK-293 (human kidney, ATCC CRL-1573) cells were grown to approximately 80% confluence according to standard cell culture techniques in T75 flasks. They were stimulated for phosphorylated protein production with 0.2-2 ug/ml anisomycin (Sigma) in serum free medium for 30 minutes. Negative control cells were incubated with serum free medium without stimulant, also for 30 minutes.

Following treatment, HEK-293 cells were centrifuged, and then medium was aspirated and replaced with MPER (Mammalian Protein Extraction Reagent, Pierce) and incubated for 15 minutes on ice with vortexing every five minutes for effective cell lysis.

Phosphatase and protease inhibitors (HALT, Thermo Fisher (Pierce) were included in the detergent cell treatment preparations. Cell lysates were aliquoted and snap frozen on dry ice, and then transferred to a -80C freezer until analysis. A BCA protein assay (Pierce) was used to determine total protein concentration of the lysates.

ViBE® Assay for the Detection of Target Analytes in Cell Lysates

Streptavidin-coated paramagnetic beads (Dynal) were coupled with biotinylated rabbit or mouse anti-analyte antibodies. Paired anti- analyte antibodies were labeled with fluorescein for ViBE assay detection reagents. These reagents were diluted for assay use in standard biological diluents to ViBE assay-specific concentrations. Assays were run in a sandwich format, with simultaneous incubation of the sample with the beads and fluorescein labeled antibody.

Cell lysate samples were prepared for the assays by thawing them on ice then clarifying by centrifugation in a microcentrifuge at 14,000 RPM for 10 minutes. Supernatants were removed from the samples and the total protein concentrations were adjusted in MPER for the assays. Serial two-fold dilutions were also prepared in MPER and subsequently assayed in triplicate. Forty microliters of sample were assayed for each dilution. The sample/reagent incubation time together was 4 hours prior to analysis on the ViBE. Elapsed time to achieve results was approximately 8 hours. Negative controls containing the bead only with the samples (no fluoresceinated antibody) were run and the ViBE response was subtracted from the sample signal readings to determine the specific assay signal.

Tumor Injection Preparation/ Injection

- Thawed BD Matrigel™ Matrix HC at 4 °C on ice overnight.
- Chilled all pipette tips, pipettes and microcentrifuge tubes overnight at 4 °C.
- Obtained A431 cells from the Cell Culture Department at TGA Sciences, Inc.
- Prepared a 1:2 dilution of cells into the Matrix on ice immediately prior to injection into mouse. The final cell concentration of the dilution was ~1 x 10^6 cells/ 100 µL.
- Using the syringe needle a subcutaneous pocket was formed by swaying the needlepoint to the right and left after needle insertion. The 10 mice were split into 2 groups, Groups C and D, with 5 mice per group.
- A volume of 100 µL of the matrix/cell dilution was injected into the subcutaneous pocket formed with the needle. The syringe was rotated during withdrawal to prevent leakage of the matrix/cell dilution.

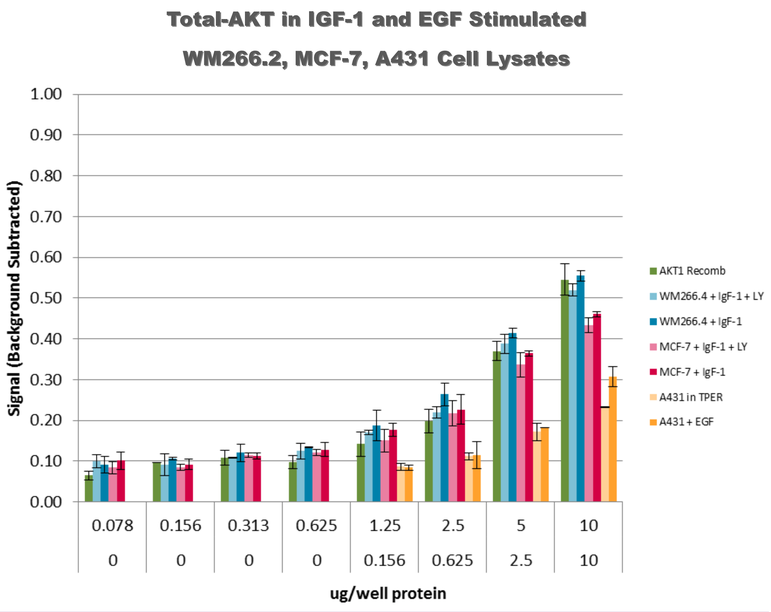
Tumor Growth Measurement

1. Immediately after injection of all 10 mice the length and width (cm) of the tumor site was measured using a caliper measuring device.
2. The tumor length and width was recorded daily. The average of the length and width was calculated for each day of measurement.
3. The tumors were harvested if the tumor average exceeded 0.5 cm in size or if the tumor site threatened to break through the skin. The harvested tumors were immediately flash frozen in liquid nitrogen and stored in 2 ml cryo- vials at -80 °C until processing.

Tumor Lysate Preparation

1. Weighed each frozen tumor into a 15 ml polypropylene round bottom conical. Recorded the weights of each individual tumor.
2. Allowed tumor samples to thaw at room temperature prior to homogenization.
3. Calculated the volume of Complete Lysis Buffer needed using the tumor weights.
4. Prepared 75 ml of Complete Lysis Buffer by adding 750 µL 100X Halt PIC and 750 µL 100X 0.5M EDTA to 73.5 ml of T-PER.
5. Added the appropriate volume of Complete Lysis Buffer to each individual thawed tumor sample.
6. Homogenized each sample using a handheld homogenizer. Allowed samples to settle at 2 – 8 °C and then re-homogenized. Repeated this step two times for each sample. The homogenizer probe was changed between each tumor sample.
7. Centrifuged samples at 3300 RCF for 5 minutes at 4 °C.
8. Aliquoted six 150 µL aliquots into 0.5 ml microcentrifuge tubes per tumor sample. Stored the remaining sample in bulk. Stored lysate samples at -80 °C until lysate processing or shipment to BioScale.

AKT Assay Development



CAPTION: To determine assay feasibility, Total-AKT measurement in several carcinoma cell line lysates is shown. Results were consistent with our expectations, with similar Total-AKT responses for treated and untreated cells.

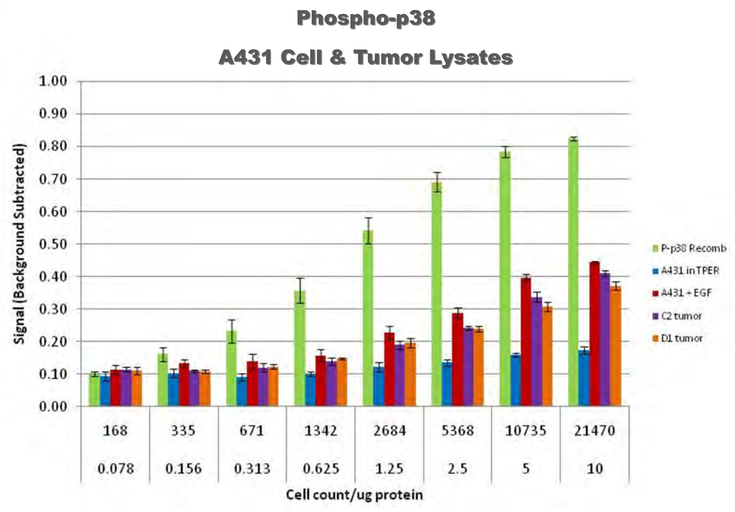
In this series of experiments, AKT assay development was done with many cell lysates including:

HEK-293	NIH3T3	WM266.2
A431	MCF-7	Several recombinant

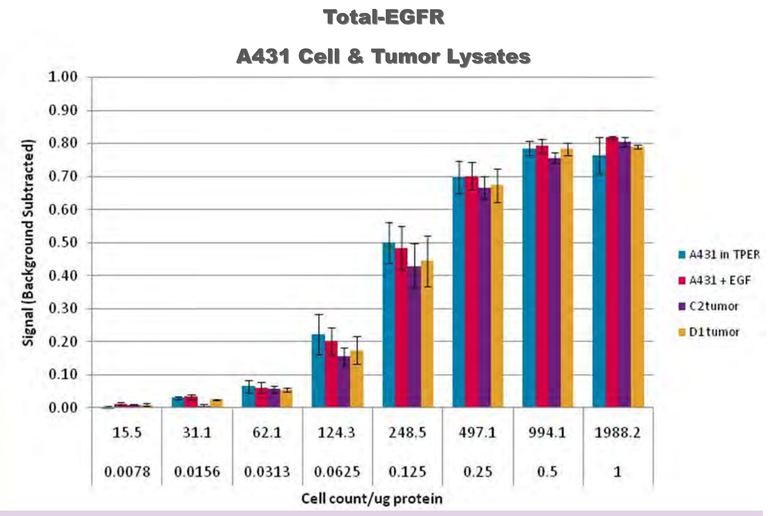
Results of the assays were promising and demonstrated differences between phospho- and Total-AKT in stimulated versus unstimulated or inhibited cell lysates. Additional studies in the HEK-293 cell line were focused on phospho– and Total-AKT assays in tumor lysates because both could be easily measured.

It was notable and encouraging in early assay development that AKT could be measured in all of the carcinoma cell lysates that we had prepared in-house and that the levels detected in treated vs. un-treated samples made scientific sense.

In Vitro versus *In Vivo* Analyte Analysis – Assay Development

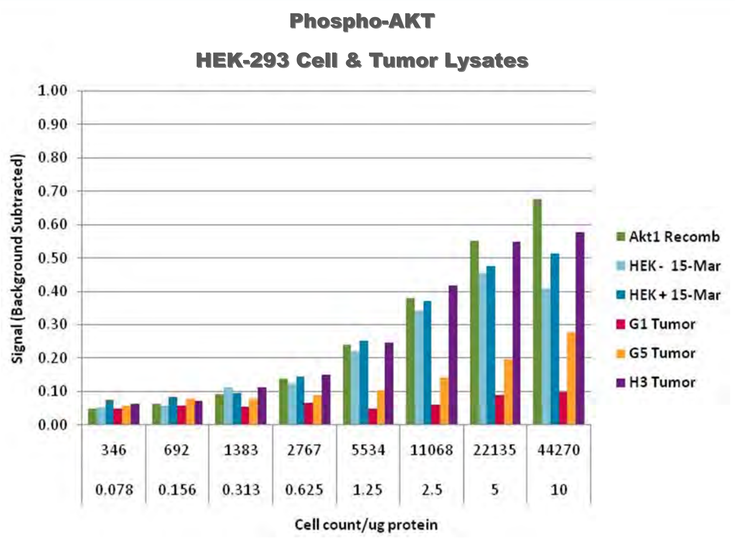


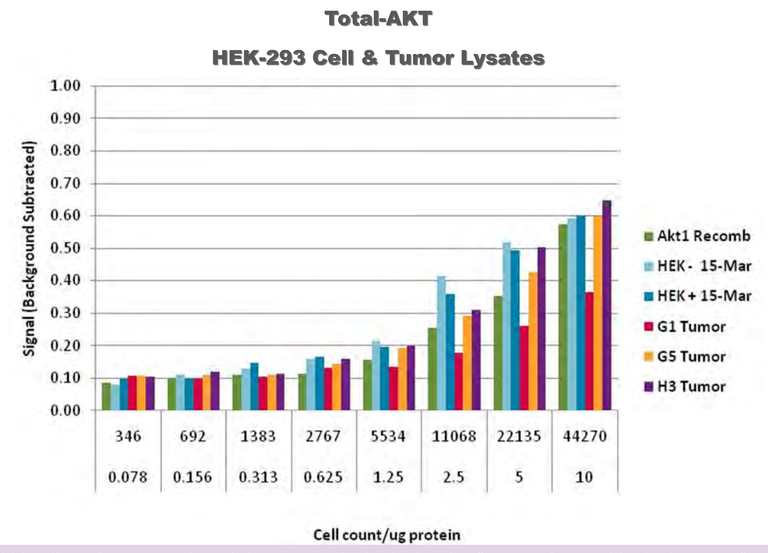
CAPTION: This graph represents a comparison of p-p38 levels in cell and tumor lysates of A431 cells. Observed are similar measurements for stimulated cells *in vitro* and tumors (no treatment) with CVs <10%, low cell numbers and *r*-p-p38 (active) as a positive control which was diluted in assay buffer.



CAPTION: Here is a comparison of Total-EGFR in A431 cell and tumor lysates, which gives expected and nearly identical measurements in the assay. Cell numbers in the hundreds can be differentiated from background by this assay.

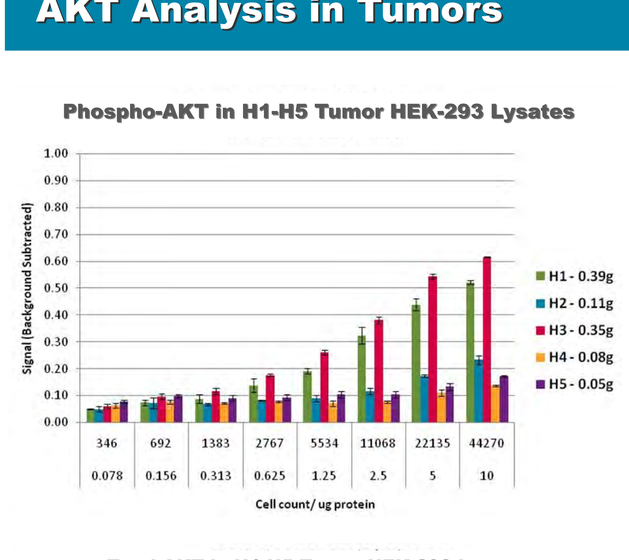
The graphs presented here demonstrate reproducible assays of natural p-p38, EGFR , p-AKT and Total-AKT in cell lysates and lysates of xenograft tumors in nude mice. Two tumor types are represented: A431 epidermal carcinoma and HEK-293 embryonic kidney cells. The lowest cell numbers detected in each cell or tumor lysate approach the 100-1000 cell range, based on the correlation between protein concentration and cell number. The measurements are reproducible with %CVs of < 10%. Observed signal levels for each of the analytes, p-p38, Total-EGFR, p-AKT and Total-AKT were comparable between cell and tumor lysates.





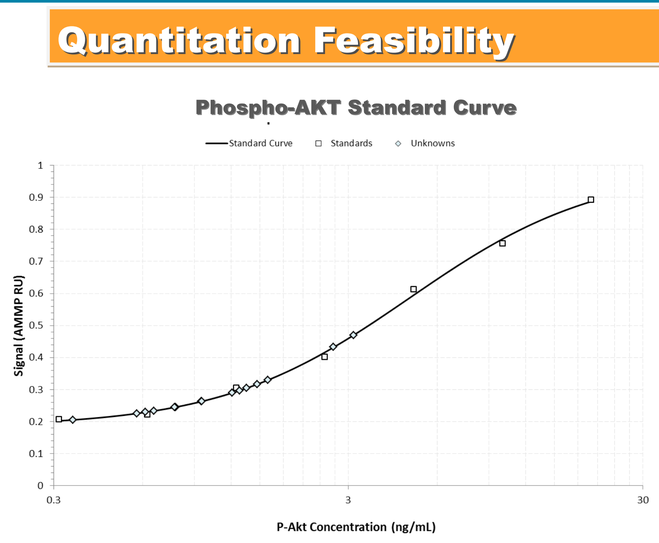
CAPTION: These graphs illustrate a comparison of levels of phospho- and Total-AKT in lysates of HEK-293 cells and tumors. There is heterogeneity in the levels of p-AKT observed in the lysates of the tumors.

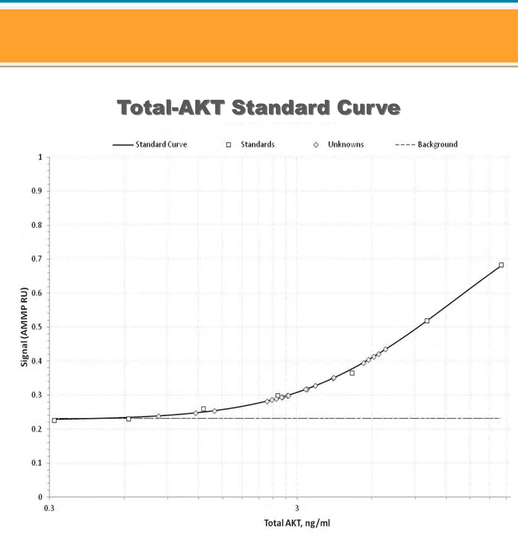
AKT Analysis in Tumors



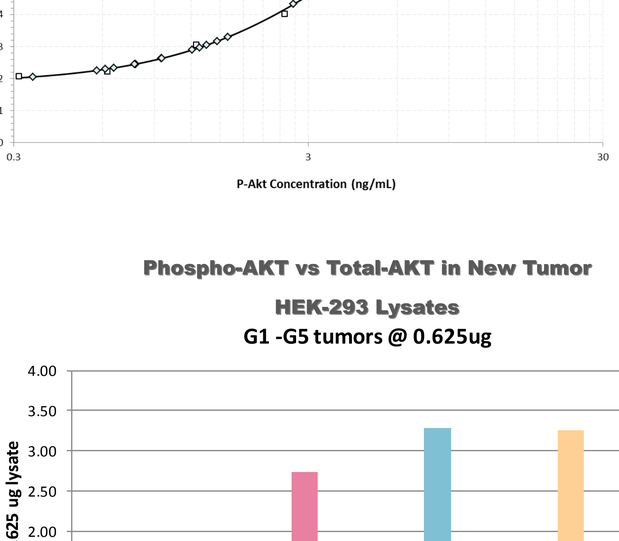
CAPTION:
Here we present results of the p-AKT and Total-AKT Assay on group "H" of the HEK-293 tumors. Within-assay reproducibility shows CVs of less than 10%. Tumors cell numbers of approximately 2000 -5000 can be differentiated from background. Both assays illustrate the heterogeneity of AKT content of tumors.

Quantitation Feasibility





Quantitation allows for assessment of the percentage of phosphorylated-AKT compared to Total-AKT in cell lysates and tumors. With assay results less effected by day-to-day variation in reagents, more robust assays have been created. An assay control from a lysate with a known signal can demonstrate that assays are performing reliably day-to-day.



Here the feasibility of quantitating phosphorylated and Total-AKT is demonstrated. The *r*-AKT(active) standard curves were generated by the ViBE, making it possible for the AKT values to be determined. Regression analysis comparing raw AMMP units with calculated concentrations for the p-AKT and Total-AKT indicates a positive relationship between the two methods, with an R squared of 0.96. Graphs include:

- Standard curves of *r*-AKT in the phospho- and Total-AKT assays.
- A comparison of phospho- vs. Total-AKT concentrations in all 10 tumors (Sets G and H, both HEK-293) as calculated, based on the standard curves. Quantitation done using a constant amount of total protein (0.625 µg) for all of the tumors. Feasibility of quantitation of these biomarkers in cell and tumor lysates was established.
- A comparison of phospho- to Total-AKT ratio calculated by raw signal (AMMP units) or as calculated from concentrations calculated by the ViBE. Regression analysis of the data presented here was performed in order to generate the R squared value and regression equation to demonstrate the relationship between the two methods numerically.

Conclusions

- We have proven the feasibility of quantifying biomarker analytes in the complex matrices of cell and tumor lysates.
- We have successfully measured native phospho- and total kinase biomarkers in xenografted tumors and the cell lines from which they were derived at nearly identical levels - a novel result that we had not previously encountered in published literature.
- The quantitation (mass/volume) of biomarker proteins using the AMMP assay format permits the elucidation of the phospho- to total kinase ratios observed in both tumor cell lines and xenografted tumors derived from them. These ratios appear to be characteristic of the individual cell lines, and will provide medicinal chemists additional criteria from which to define appropriate therapeutic targets for drug discovery.
- The correlation of quantitated biomarker values vs. raw AMMP signal units has been demonstrated to be significant in this study, with an R squared of 0.96 and a regression equation of y=1.9x+0.13 for the data sets being compared.
- The relationship between total protein and cell numbers allows for an assessment of sensitivity based on cell numbers (<1000 cells/measurement). This is advantageous when analyzing tumor cell biopsy samples where cell numbers are generally low and allows for more screening options with limited sample. The numbers of cells required for the determinations are consistent with those available from fine needle aspiration of *in situ* tumors, thus permitting the potential for "finger-printing" a specific tumor and evaluating multiple chemotherapeutic regimens against the tumor.

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