Development of Acoustic Membrane MicroParticle (AMMP™) Assays for Rapid and Sensitive Detection and Quantitation of BioMarkers for Complex Biological Matrices and Comparisons to Traditional Technology

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Abstract

Biomarker immunoassays are revolutionizing biopharmaceutical science and laboratory medicine. These assays have made possible the measurement of minute amounts of proteins in a diverse array of biologically relevant samples. The sensitivity and specificity of traditional immunoassays is frequently limited by endogenous and exogenous interferences that cannot be eliminated and researchers struggle to identify and eliminate their effects.

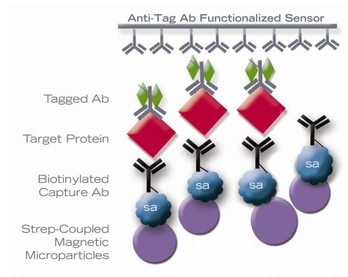
The purpose of this work is to demonstrate the performance of newly emerging technology: the ViBE Workstation and AMMP assays on biomarker proteins found in several complex biological matrices. Using an assortment of preclinical and clinical research samples from cell culture, tumor lysates, amniotic fluid and urine, the feasibility of the ViBE workstation was tested for detection and quantitation of pharmacodynamic, GADD-34, IL6, Kidney injury (KIM-1) and intrauterine inflammatory biomarkers. The ViBE performance was evaluated and compared with established ELISA techniques, Western blots and commercial tests such as Luminex, Alphascreen® and SELDI-TOF-MS.

Results:
To meet today's biomarker research needs, protein scientists require an assay and sample prep platform that can rapidly and consistently deliver accuracy, reproducibility, sensitivity and assay performance across a broad dynamic range and with difficult and complex sample matrices. These data demonstrate that samples derived from a variety of sources can be readily analyzed using the ViBE Platform using the open AMMP assay technology. In all of these assay types, these data show that the ViBE produces results comparable or better than established techniques, eliminates the labor intensive effort of Western blot procedures and does not exhibit optical and chemical interferences derived from lysates, tissue culture, *in vivo* xenograft tumors, amniotic fluid, and urine that is observed in optical detection techniques.

Technology and Instrumentation

BioScale's ViBE BioAnalyzer and ViBE Workstation powered by Acoustic Membrane Microparticle (AMMP™) assay technology were assessed in the analysis of protein biomarkers found in several complex biological matrices. Comparison data using traditional assay methods such as Western Blot and ELISA were used to determine comparability.

AMMP is the unique integration of magnetic microparticles, a universal sensor and acoustic detection creating a protein detection technique that is highly robust and versatile. AMMP assays use a traditional homogeneous assay format with a proprietary detection technology providing results with fewer sample limitations, simple operation and fast assay development.



Phospho-Akt in Cell Culture & Tumor Lysates (Xenografts)

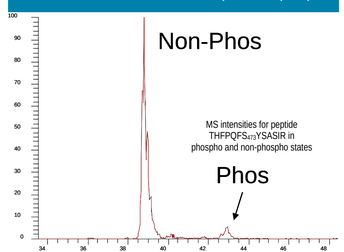
Monitoring Inhibition from Tissue Culture and Mouse Xenografts

Protein kinase AKT, 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. Activation of Akt, through phosphorylation of both Thr308 and Ser473, 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.

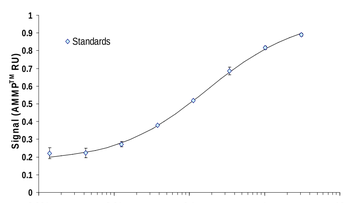
Millennium Pharmaceuticals sought a strategy for monitoring the inhibition of pSer473-Akt using both tissue culture and mouse xenografts. A pair of commercially available monoclonal antibodies against both pSer473-Akt and pan-Akt were selected and each affinity tagged with either biotin or fluorescein. Using the ViBE™ Protein Analysis Platform with AMMP™ Assay technology, 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 with Li-Cor Western Blot analysis.

Establishing standard curve of pSer473-Akt with AMMP Assay on the ViBE Bioanalyzer
Recombinant GST-tagged-Akt2 was phosphorylated in vitro with mTORC2 complex. The percentage of phosphorylation was analyzed using mass spectrometry. This batch of pSer473-Akt was used to establish a standard curve titrated over an eight point three-fold dilution series in buffer. 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.

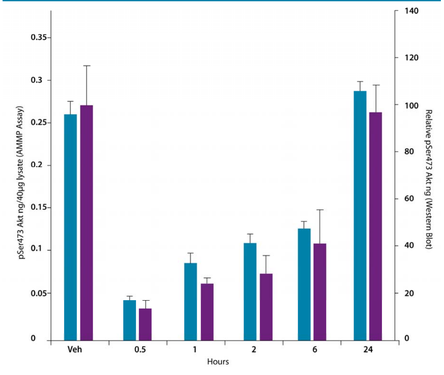
in vitro Phosphorylated Recombinant GST-Akt2 (Mass Spec)



pSer473-Akt Standard Curve (AMMP Assay)



pSer73 Akt Inhibition from Xenograpfts Following Treatment with a pan-PI3K Inhibitor AMMP Assay vs Western Blot



Taken form published research for phospho Akt and Gadd34: Z.-H. Yan et al., Analysis of two pharmacodynamic biomarkers using acoustic micro magnetic particles on the ViBE Bioanalyzer, Anal. Biochem. (2010), doi:10.1016/j.ab.2010.11.012

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 or Li-Cor Western Blot
Three tumor-bearing mice were treated orally with 100 mg/kg TGX221 compound and WM266.4. Xenograft samples excised at each designated time point. Protein concentrations were determined by bicinchoninic acid (BCA) for either vehicle- or drug-treated samples. The same complementary pairs of Akt/pAkt antibodies as described above were used for the AMMP assay and a single anti-pSer473-Akt antibody for Li-Cor Western Blot analysis. The patterns of inhibition were equal for the 2 methods, showing an sharp reduction of ~90% of pSer473 at 30 min followed by a slow recovery over the course of 24h back to basal levels. However the Li-Cor Western Blot results were normalized relative to the vehicle treatment group at 100%, for ease of data analysis, whereas the data analysis for the AMMP assay produced quantitative results for the biomarker relative to the sample input (40µg tumor lysate/analysis). Results shown are the averages and standard deviations of replicate determinations representative of at least duplicate experiments with standard errors of less than 10%.

Results

The results show clearly that the AMMP Assay 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. Millennium also drew the following conclusions from this study:

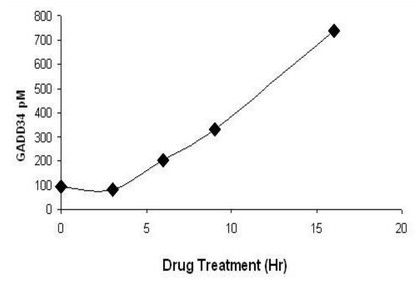
1. Samples derived from a variety of sources can be readily analyzed using the AMMP Assays and the ViBE™ Bioanalyzer producing the same quality results from recombinant, tissue culture or *in vivo* tumor xenografts.
2. Unlike Li-Cor Western Blot the ViBE™ Bioanalyzer affords rapid reproducible and quantitative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

Gadd34 Induction by Proteasome Inhibition from Cell Culture & Tumor Lysates (Xenografts)

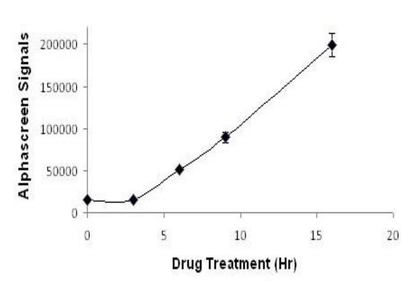
Monitoring Protein Expression in a Xenograft Tumor Lysates

Sample effects can make developing and using traditional detection schemes difficult and sometimes impossible on critical time lines. Gadd34 (Growth Arrest and DNA Damage protein 34) is a stress-induced protein implicated in the control of protein synthesis and apoptosis. Under normal non-stressed conditions Gadd34 is a rapidly degraded protein through the ubiquitin-proteasome pathway. Because proteasome inhibition by bortezomib or its analogs induces Gadd34 accumulation, Millennium Pharmaceuticals sought to develop a strategy for monitoring increases in protein levels. In their study, a complementary pair of rabbit monoclonal antibodies was developed against recombinant Gadd34. These antibodies were then affinity tagged and used to generate a standard curve with recombinant Gadd34. Cell culture derived HCT116 or CWR22 xenograft tumor lysates either treated or untreated with proteasome inhibitors were analyzed using an AMMP assay on the ViBE Bioanalyzer and compared to results obtained with either LiCor® Western Blot or AlphaScreen®.

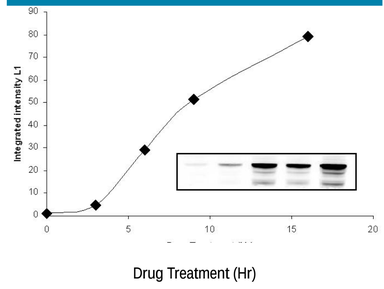
AMMP™ Assay on ViBE™ Platform



AlphaScreen®

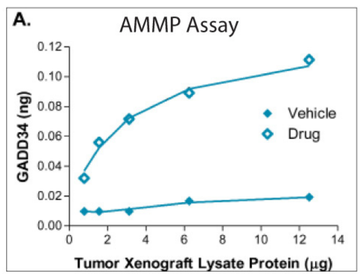


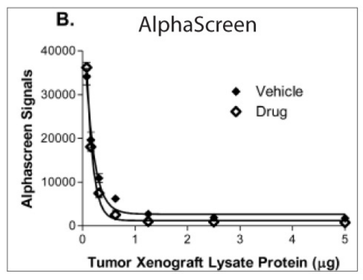
LiCor® Western Blot



Induction of Gadd34 of HCT116 derived cell culture lysates in the presence or absence of boronate proteasome inhibitor as analyzed by either AMMP Assay or AlphaScreen® or Western Blot LiCor®.

HCT116 cells were cultured in the presence or absence of 3 nM of a bortezomib analog and lysed in mPER buffer at the times indicated. Protein concentrations were determined by BCA and normalized for sample addition for each assay. Results shown are representative of at least duplicate experiments with standard errors of less than 10%.





Induction of Gadd34 derived from CWR22 xenografts in the presence or absence of a boronate proteasome inhibitor as analyzed by either ViBE™ BioAnalyzer or AlphaScreen®
Tumor bearing mice were treated iv with a bortezomib analog and tumor xenograft samples excised and homogenized following a 16 hr dose. Protein concentrations were determined by BCA. In the case of the AMMP assay, the signal was normalized to the rGST-GADD34 signal using ViBE software. Note the quenching of the AlphaScreen signal with increasing tumor µg of protein added to the assay. Only the analysis of the AMMP Assay samples produced meaningful results for the detection of Gadd34 across the lysate titration range given that significant induction could be detected above vehicle at all lysate concentrations, with the average induction being constant at ~6-fold between 1.5 and 12.5 µg of tumor lysate. When Identical samples were analyzed with AlphaScreen, there was a clear reduction of signal across the titration range that interfered severely with the detection of Gadd34 for both treatment groups, clearly illustrating the limitations of this method with mouse xenograft-derived samples. Results shown are representative of at least duplicate experiments with standard errors of less than 10%.

Results

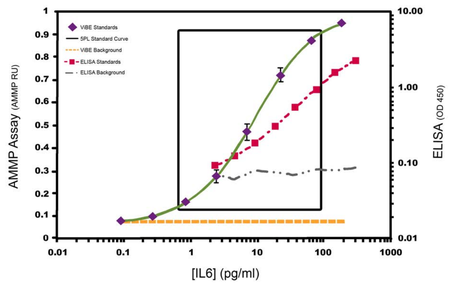
Millennium concluded that the use of the ViBE™ Platform using AMMP Assay technology eliminates the labor intensive effort of Western Blot analysis and, furthermore, is devoid of the optical and chemical interferences derived from lysates of xenograft tumors observed with AlphaScreen®.

IL-6 & Intrauterine Inflammation Markers in Amniotic Fluid

Sensitive Interleukin-6 Quantitation in Amniotic Fluid

Concentrations of IL-6 in amniotic fluid have been linked to women with intrauterine infections and is postulated to be an inflammatory marker of early onset neonatal sepsis (EONS). Recent advances in proteomics of amniotic fluid including ELISAs and surface enhanced laser desorption mass spectrometry (SELDI-TOF-MS) have generated novel research and diagnostic applications to better understand the mechanism of maternal and fetal response to intrauterine infection. Although sensitive, SELDI-TOF-MS studies require expensive equipment, highly trained operators and generally supply a very low sample through-put. Current techniques such as ELISA take a long time to process and require much hands-on time or micro-organism culture takes 4-5 days to get results. The purpose of this study was to evaluate the ViBE Protein Analysis Platform using AMMP Assay technology to determine their performance and adaptability in measuring IL-6 in clinical amniotic fluid samples from a 100 patient non-hierarchical cohort and compare the results versus using a standard eBiosciences ELISA. This is a portion of the currently unpublished study.

Calibration Curve Intraleukine-6 (IL-6) AMMP Assay vs ELISA

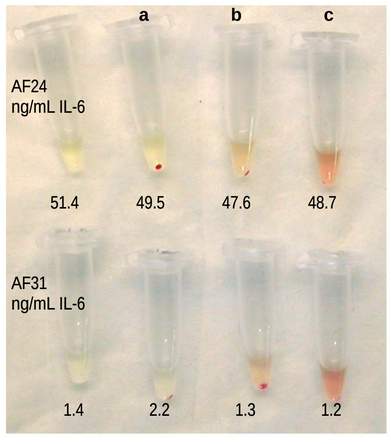


The linear range of IL-6 AMMP™ assay is 0.3 to 66.7 pg/ml IL-6. The working range of quantitation, defined by calibrator CV of less than 20% is 0.8 to 66.7 pg/ml). In contrast, the lower limit of detection (LLOD) of the colorimetric ELISA is 2.3 pg/ml. Therefore the lower limit of quantitation (LLOQ) of the IL-6 AMMP™ Assay (0.8 pg/ml) is three times more sensitive than the equivalent ELISA (2.3 pg/ml)

Spike Recovery IL-6 Amniotic Fluid

	AMMP Assay		
	ng/ml IL-6 Recovered	ng/ml IL-6 Expected	% Recovered
AF11 (100%)	203.4		
AF19 (100%)	320.5		
AF24 (100%)	67.1		
AF09 (100%)	27.3		
AF14 (100%)	2.1		
AF28 (100%)	2.6		
AF11:AF09 (10%:90%)	44.3	44.9	98.5%
AF11:AF14 (10%:90%)	22.1	22.2	99.5%
AF11:AF28 (10%:90%)	24.5	22.7	108.1%
AF19:AF09 (10%:90%)	63.5	56.6	112.1%
AF19:AF14 (10%:90%)	34.0	33.9	100.3%
AF19:AF28 (10%:90%)	33.6	34.4	97.7%
AF24:AF09 (10%:90%)	35.1	31.3	112.0%
AF24:AF14 (10%:90%)	9.3	8.6	108.5%
AF24:AF28 (10%:90%)	10.4	9.1	114.9%

Two AF samples with high (AF11 and AF19), medium (AF9 and AF24), and low (AF14 and AF28) levels of IL-6 were selected for spike recovery. One part of AF11, AF19 and AF24 were spiked into 9 parts of AF9, AF14 and AF28 for a total of nine spiked samples. The six neat samples and 9 spiked samples were tested for IL-6 concentration at a 1:10,000 dilution. The recoveries of these spiked samples ranged from 97.7%-114.9 %.



Quantitaion of Contaminated Samples

AF24 (57.6 ng/mL IL-6) and AF31 (1.3 ng/mL IL-6) were spiked with whole maternal blood (a), hemolyzed maternal blood (b) or hemolyzed cord blood (c). The samples were then measured for IL-6 concentration on the ViBE™ using an AMMP Assay. No significant difference in the quantitation of IL-6 was observed with or without blood contamination.

Results

A linear relationship was observed in both the AMMP assay and eBioSciences ELISA however the AMMP assay was three times more sensitive in measuring IL-6. The data from both correlate well (p-value<0.0009). The % difference of duplicate reading of AF samples containing > 10 ng/mL is less than 10%. In the 100 AF sample cohort using a 1:10,000 dilution run in duplicate, the ViBE screening was completed in a day and a half while the same screening with the eBiosciences ELISA took five days. This study concludes that the AMMP assay is linear, accurate and precise, has a larger linear range than ELISA and is accurate even in the presence of whole blood contaminants.

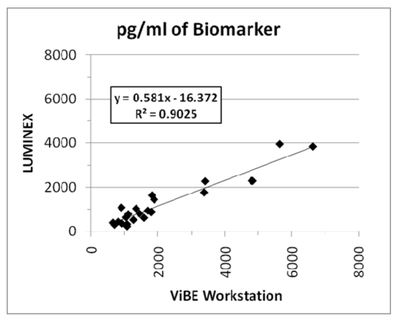
Kidney Injury Biomarker (Kim-1) in Urine

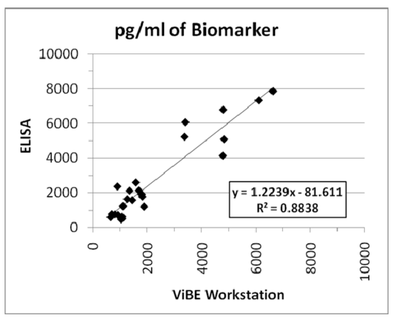
Rapid Biomarker Analysis in Urine

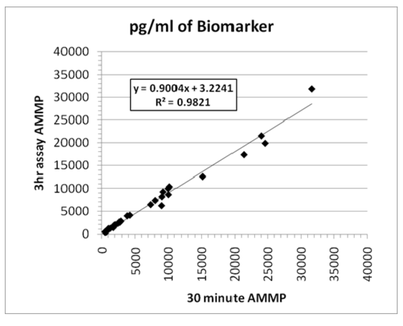
Presently, acute kidney injury is monitored by measuring an increase in Blood Urea Nitrogen (BUN) and creatinine. Unfortunately changes in BUN are usually only detectable after irreversible damage has been caused to the kidney. Urinary kidney injury molecule (KIM-1) is a sensitive quantitative biomarker for early detection of kidney tubular injury. The KIM-1 kidney biomarker is under study because this biomarker rapidly increases in urine and can indicate kidney damage when intervention is still an option. A study was done using the ViBE Workstation running an AMMP Assay versus a Luminex creatinine and ELISA assay to evaluate each technology and its feasibility to be used in a rapid 40 minute analysis.

Three Assay Methods

Luminex	ELISA	ViBE Workstation using AMMP Assay
• Wash beads	• Coat plate overnight, Wash	• Add reagents to diluted samples
• Add sample dilutions	• Add sample dilutions	• Incubate on deck
• 3 wash steps	• 4 wash steps	• Automated sample analysis
• Add sample to instrument	• Read plate	RESULTS INDEPENDENT OF INCUBATION TIME
Incubation Time: 3 hours	Incubation Time: 3 hours	Incubation Time: 30 Minutes vs 3 Hours







Results

There are challenges in dealing with complex samples, such as urine when analyzing protein biomarkers. Using the methodologies defined above, the ViBE Workstation using AMMP Assay technology demonstrated comparable data to the Luminex and ELISA assays with three hour incubation times, however, the ViBE also achieved the same results with only a 30 minute incubation time offering a significant workflow benefit.