Development of Applications of Acoustic Membrane MicroParticle Assay Technology for Translational Medicine

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Abstract

The detection and study of proteins and their interactions in small samples of cells or tissues is an ongoing problem in cancer research. Recent advances in the early detection of many cancers has resulted in smaller sized tumors for biopsy and evaluation. This has created a need for ultrasensitive assays for pathway related proteins. One mission for the Antibody and Protein Purification Unit (APPU) of the National Cancer Institute has been to evaluate emerging technologies and create new applications for these technologies. Using Acoustic Membrane MicroParticle (AMMP) assays on the ViBE Workstation (BioScale, Lexington, MA) for solution phase immunoassays, the APPU has developed several applications which suggest the great utility and breadth of applications for this technology in translational research. These assays use a homogenous, sandwich assay format with paired antibodies, one coupled to a magnetic bead, and the analyte forming a complex which interacts with a vibrating membrane sensor. The assay measures the resonant frequency shift of the membrane caused by the protein interaction -- without matrix interference or signal scatter seen in optical techniques. A highly sensitive assay for Prolactin showed no serum interference which is a common problem in optical immunoassays for circulating peptide hormones. An assay for the important marker c-Met required less than 125 nanograms of cell lysate for detection and quantitation. An AMMP assay was further able to quantitate c-Met on the surface of HepG2 cells using an antibody capture method. In an application for detection and quantitation of a member of the MAPK kinase family, an ultrasensitive assay for the detection of MEK1 was developed as well as a highly specific solution phase assay for pMEK1 (S218/S222) as shown by cognate phosphopeptide inhibition. And finally in an application which we believe is truly unique, an in solution heterodimer assay for the ERK2/MEK1 interaction was shown to be susceptible to inhibitor treatment. In conclusion, the AMMP technology was able to perform a wide range of applications required by translational research studies. The assays were extremely sensitive and reproducible requiring nanogram quantities of lysate proteins. The ability to measure protein/protein interactions in solution phase is most appealing for drug development and evaluation of drug effect in targeted therapies.

Introduction

Detection and quantitation of protein biomarkers are essential for elucidating cancer biology and for efficient clinical diagnosis. In oncology drug development, determining biological responses to drug-on-target interactions are critical parameters. The development of custom assays for such unique proteins is often limited to time-consuming manual techniques using optical detection and quantitation procedures, such as ELISA or Western blots. The complexity of development on alternate platforms, and the difficulty of using conventional assays to detect biomarkers in complex biological matrices contribute to this limitation in assay development. The Acoustic Membrane MicroParticle (AMMP) assay technology (BioScale Inc., Lexington, Mass.), performed on the ViBE protein analysis platform (BioScale Inc.), permits the use of a single technology for detecting oncology biomarkers in serum, cell lysates, cell surfaces and a wide variety of other sample types.[1-2]

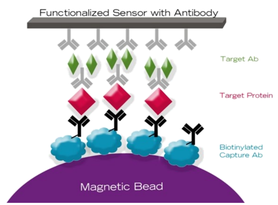
AMMP assays may offer significant advantages over conventional approaches to assay development — most importantly, the non-optical detection of low-abundance biomarkers in complex samples with minimal sample manipulation. Other reported advantages of the AMMP technology include improved sensitivity and reproducibility, less manual sample manipulation, the capability of walk-away operation, reduced consumption of critical samples and antibodies, and the ability to measure proteins in their natural states.

AMMP technology assesses analyte concentration by measuring changes in the oscillations of a piezoelectric sensor as antibody-labeled magnetic microparticles bind to the sensor surface. The biosensor membranes are resonant devices that change their oscillation frequency in direct proportion to mass accumulated on their surfaces, thus permitting rapid and efficient determination of analyte concentration. These AMMP assays have been used to analyze pharmacodynamic biomarkers for inhibition of the proteosome and phosphoinositol 3-kinase.[2] In that context, the AMMP assay proved to be a robust method for measuring recombinant protein standards as well as endogenously derived proteins from both tissue culture and mouse xenograft tumor lysates.

We examined the suitability of the AMMP assay technology for detecting oncology biomarkers in extra- and intracellular environments, as well as on cell surfaces. In the studies presented here, we utilized this technology to meet several objectives: to detect and quantify a secreted biomarker in human serum; to specifically and sensitively measure a cell surface biomarker in cell lysates, live cells, and fixed cells using antibodies directed at extracellular domain antigens; to detect kinase phosphorylation events on specific targets in cell lysates; and to utilize a peptide-specific drug to measure the formation and disruption of protein dimers, both with recombinant proteins and in cell lysates.

Assay Methodology

AMMP® Assay Format



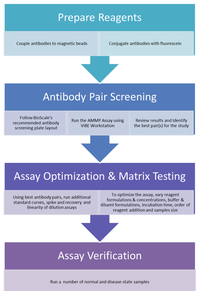
An AMMP assay measures antigen concentration by measuring changes in oscillation frequency caused by the binding of magnetic microparticles to the sensor surface. It consists of four major components: (1) a ViBE cartridge that contains a functionalized eight sensor chip (2) antibody labeled magnetic microparticles, (3) Fluorescein tagged antibody (Fl-Ab) that binds to the surface of the functionalized chip (4) samples containing the analyte of interest. BioScale's biosensor is a resonant device that contains eight individual membranes with separate fluidic paths. The membranes change oscillation frequency in response to mass accumulating on the surface, and this frequency change is used to quantitate the amount of added mass.

Assay Development Process

The assay development process enables rapid creation and optimization of each assay. Following the selection of analyte, the source of the natural analyte (e.g., cell, tumor, serum, plasma, urine or other biological fluids) and selected antibodies to the analyte from either a commercial source or custom-sourced, the assay can be developed.

The process is streamlined with each of these cytokine assays developed and verified in 3-7 days as compared to months when using other assay and western blot techniques.

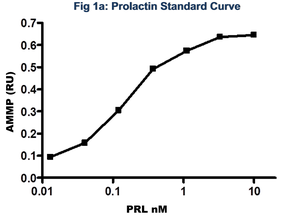
A full verification study includes multiple lots of reagents and cartridges with different operators over multiple days. This type of study measures: precision (LOD, LOQ, range), accuracy (recovery & linearity), specificity and cross-reactivity, in matrix disease state samples, and technology comparisons.



Results

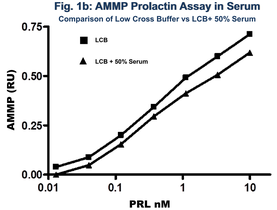
Development of Prolactin Assay

Fig 1a: Prolactin Standard Curve



Caption Figure 1 A: Rabbit polyclonal antibody specific for PRL was generated by Primm Antibody Inc.(Boston, Mass) was labeled with either biotin (for conjugation to streptavidin-magnetic beads) or FITC for use with the AMMP assay. For standard curves of PRL, recombinant PRL was diluted into Bioscale diluent and titrated over a 3-fold dilution series to the indicated concentrations.

Fig. 1b: AMMP Prolactin Assay in Serum
Comparison of Low Cross Buffer vs LCB+ 50% Serum

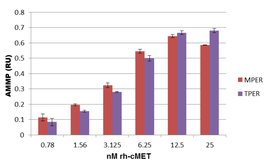


Caption Figure 1b: For the evaluation of serum interference in the PRL assay, recombinant PRL was diluted in 3-fold dilutions in Low Cross Buffer (Candor Biosciences GmbH) with or without 50% human serum. The assay was performed using the same antibody loadings as in Figure 1a. The PRL assay was minimally inhibited by the high serum concentration.

Development of cMET Assay

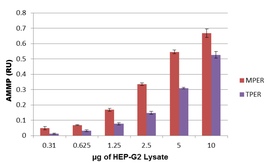
10-Fold Increase in Assay Sensitivity Achieved

Fig. 2a: cMET Assay Standard Curve



Caption Figure 2a: Recombinant c-Met (HGFR/cMet fc chimera, R&D Systems, Cat33 38-MT) was titrated in 2-fold dilutions in TPER or MPER buffers (Pierce, Rockford, IL) diluted 1:1 in BioScale diluent. An anti-human HGF rh-cMet monoclonal antibody (R&D Systems, MAB3581) was labeled with biotin (for conjugation to streptavidin-magnetic beads) and paired with an anti-human HGFR/cMET antibody (R&D AF276) to FITC for use with the AMMP assay as directed by the manufacturer. All assays were done in triplicate and the error bars show the standard deviations.

Fig. 2b: cMET Expression in HepG2 Lysates



Caption Figure 2b: Detection of cMet in HepG2 TPER or MPER extracted cell lysates was performed using the same reagents for Figure 2a. It appears that MPER extraction is more efficient. All assays were done in triplicate and the error bars show the standard deviations.

Fig. 3a: Optimized cMET Assay Standard Curve

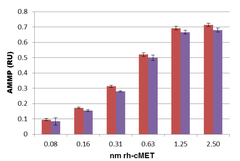
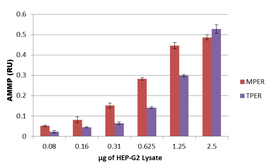
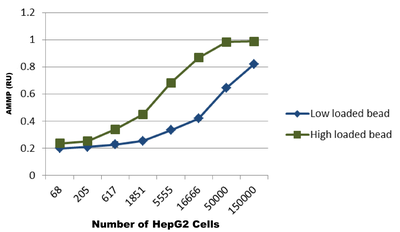


Fig 3b: Optimized Detection of cMET in HepG2 Lysates



Caption Figure 3a and 3b: Optimization of antibody loading on the capture beads can lead to assays with even greater sensitivities than those seen in Figures 2a and 2b. By the addition of 150 pg/well over that recommended in the manufacturer's instructions a 10-fold increase in assay sensitivity was achieved. As seen in Figure 3a the assay again demonstrates no difference between the two extraction reagents. And as shown in Figure 3b, cMet was detectable in as little as 80 ng of HepG2 lysate protein. All assays were done in triplicate and the error bars show the standard deviations.

Fig. 4: cMET Expression on Intact HepG2 Cells

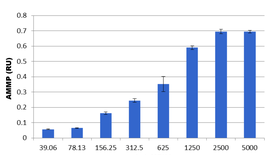


cMET Measurement Utilizing Fewer Than 1000 Cells

Caption Figure 4: AMMP assays can use different bead loading densities to adapt to the availability of sample amounts. cMet detection on the surface of HepG2 cells could be varied with the loading density of biotinylated capture antibody. cMet binding curves using the same amounts of high or low loaded beads and a titrated amount of HepG2 cells is shown in Figure 4. This feature can be important when utilizing samples with high or low amounts of cell surface expressed ligands as the assay can be adjusted to yield data from as little as 1000 cells per well in the assay.

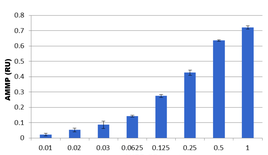
Quantitation of MEK1 in HepG2 Lysates

Fig. 5a: rh-MEK1 Assay Standard Curve



Caption Figure 5a: Generation of a standard curve for the AMMP assay of MEK1 protein is shown in Figure 5a. Baculovirus-produced MEK1 (Invitrogen) was titrated over a 2-fold dilution series to the indicated concentrations in MPER diluted 1:1 with PBS + 1%BSA diluent. MEK1 assay was constructed by biotin-labeled anti-MEK1 c-term antibody (Epitomics, 1235) conjugated to a streptavidin bead and paired with a fluorescein-labeled anti-MEK1 antibody (Cell Signaling Technology, 2352). All assays were done in triplicate and the error bars show the standard deviations.

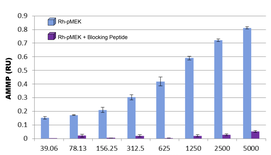
Fig. 5b: MEK1 Expression in HepG2 Lysates



Caption Figure 5b: Logarithmically growing HepG2 cells were lysed in TPER buffer and protein concentrations were determined using the Pierce BCA Protein Assay kit (Thermo Scientific) according to manufacturer's instructions. Lysates were titrated over a 3-fold dilution series to the indicated total protein amounts in 50/50 MPER/PBS 1%BSA buffer. The same MEK antibody pairing as in the previous experiments was employed for use with the AMMP assays. All assays were done in triplicate and the error bars show the standard deviations. MEK1 could be detected in as little as 20 ng of HepG2 lysate protein.

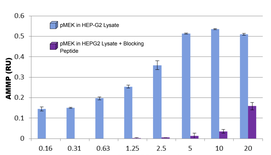
Quantitation of pMEK in HepG2 Lysates

Fig. 6a: pMEK Assay Standard Curve
Specificity of pMEK with and without blocking peptide



Caption Figure 6a: Figure 6a shows the detection of recombinant pMEK1 S217/S221 (R&D Systems) in lysates with and without addition of a pMEK-specific peptide used to immunize rabbits for production of the pMEK antibody. An anti-MEK N-terminus antibody (Cell Signaling, 2352) was labeled with FITC and an anti-pMEK S217/S221 rabbit monoclonal antibody (Cell Signaling, 9154) was labeled with biotin and conjugated to streptavidin-magnetic beads for use with the AMMP assay. A pMEK specific peptide was added to the indicated samples to a final concentration of 2.5nM. All assays were done in triplicate and the error bars show the standard deviations. The EC 50 in the assay was 927.5 pg/mL and there was no detectable binding activity seen at all data points in the presence of the inhibitor.

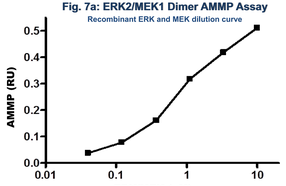
Fig. 6b: pMEK Expression in HepG2 Lysates
Specificity of pMEK with and without blocking peptide



Caption Figure 6b: Logarithmically growing HepG2 cells were lysed in MPER buffer and protein concentrations were determined using the Pierce BCA Protein Assay kit (Thermo Scientific). Lysates were titrated over a 3-fold dilution series to the indicated total protein amounts in 50% MPER buffer. A pMEK specific peptide was added to the indicated samples to a final concentration of 2.5nM. All assays were done in triplicate and the error bars show the standard deviations. The EC50 for the cell lysate assay was 2.08 ug/well. This assay appears to be very sensitive. **There have been no reports in the literature for an assay that specifically measures pMEK1(S218/222) at this level of sensitivity.**

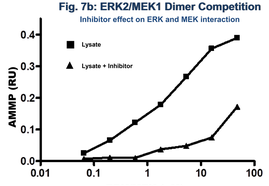
ERK2/MEK1 Heterodimer Measurement

Fig. 7a: ERK2/MEK1 Dimer AMMP Assay
Recombinant ERK and MEK dilution curve



Caption Figure 7a: The generation of an assay to detect ERK2/MEK1 heterodimers was developed using Baculovirus-produced MEK1 (Invitrogen) and ERK2 (Protein One incubated together at a concentration of 10nM, then titrated over a 3-fold dilution series in buffer recommended by the manufacturer. An anti-MEK C-terminus rabbit monoclonal antibody (Epitomics) was labeled with FITC and an anti-ERK rabbit polyclonal antibody (Cell Signaling) was labeled with biotin then conjugated to streptavidin -magnetic beads.

Fig. 7b: ERK2/MEK1 Dimer Competition
Inhibitor effect on ERK and MEK interaction



Caption Figure 7b: Inhibition of ERK2/MEK1 heterodimers in HepG2 lysates. The antibody reagents described in Figure 7a were used to detect ERK2/MEK1 heterodimers in HepG2 lysates. Inhibition of these heterodimers was performed by addition of a ERK peptide that competes with ERK for the binding site on MEK was added to the indicated samples to a final concentration of 200 nM. At lower concentrations of cell lysate there was significant inhibition, indicating that the assay was specific for the heterodimer.

Conclusions

Our preliminary experience with the AMMP technology and the ViBE Workstation has allowed the implementation of the following:

- A specific assay for Prolactin in media with high concentrations of serum.
- An Assay for c-Met recombinant protein which was compatible with cell or tissue extraction reagents. The assay was able to measure c-Met in HepG2 cell lysates.
- An ultra-high sensitivity c-Met assay which was able to measure c-Met in as little as 125 nanograms of cell lysate.
- A whole cell assay for a cell surface protein using a detection antibody as a cell capture reagent. This assay format may have applicability for the development of antibodies to cell surface determinants.
- An assay for the measurement of a member of the MAPK kinase pathway, MEK1.
- An assay determined to be specific for pMEK1 (S218/S222) by cognate phosphopeptide inhibition.
- Development of an *in solution* heterodimer assay using active recombinant proteins. The ERK2/MEK1 heterodimer was shown to be susceptible to inhibitor treatment. This application may have great applicability for drug development.

References:

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2 Yan ZH, Madison LL, Burkhardt A et al. Analysis of two pharmacodynamic biomarkers using acoustic micro magnetic particles on the ViBE bioanalyzer. *Anal Biochem* 410(1): 13-18. 2011.

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