ADEPTRIX

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Targeted Proteomic Assays to Measure Conserved Phosphorylation Sites in Protein Isoforms using Affinity Bead Assisted Mass Spectrometry (Affi-BAMS™) and MALDI MS

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INTRODUCTION

Proteomic studies often employ multi-dimensional analytical methods such as nano-LC-ESI-MS/MS to simplify sample complexity and reduce the wide dynamic range of proteins within the biological specimen. The time and expertise required to implement LC-MS methods can often be a barrier to adopting targeted proteomic methods for a particular translational research program. In this study, we present the use of a novel method that integrates immuno-affinity purification (IAP) with Matrix Assisted Laser Desorption Mass Spectrometry (MALDI-MS) for targeted proteomics of cultured cells to monitor protein isoforms, ERK1 & ERK2. The proprietary method, called BAMS™ (Bead Assisted Mass Spectrometry), can be used to monitor 1 – 100's of protein targets in a single assay. The assay is conducted in a microarray format, which can accommodate up to 2286 assays on a single MALDI slide, with as little as 10 µg of total protein, making it an ideal analytical platform for biomarker screening of cell cultures, tissues, liquid biopsies and primary cells.

METHODS

Human gastric cancer cells (MKN45) were homogenized in standard urea lysis buffer to extract soluble cellular proteins, which were then reduced and alkylated prior to protease digestion and subsequent C18 purification. Purified peptides were reconstituted in PBS for affinity capture of target peptides using Affi-BAMS beads. For each bead, captured target peptides were eluted within a restricted 500 micron area onto an ITO coated glass slide in the presence of CHCA matrix for MALDI MS analysis using a Bruker Autoflex Speed. Spectra were acquired over the mass range of 0.8 to 7.0 kDa using 10000 laser shots (consuming less than 5% of the sample) in positive ion mode at a laser power of ~ 25%. Calibration of the mass spectra was achieved using a calibration solution comprised of C18 purified tryptic digest of bovine serum albumin.

CONCLUSION

The Affi-BAMS assay platform technology enables targeted proteomics of proteins, post-translational modifications and protein isoforms by integrating highly specific antibody capture on high capacity magnetic beads along with the sensitivity and resolution of MALDI mass spectrometry for rapid biomarker screening.

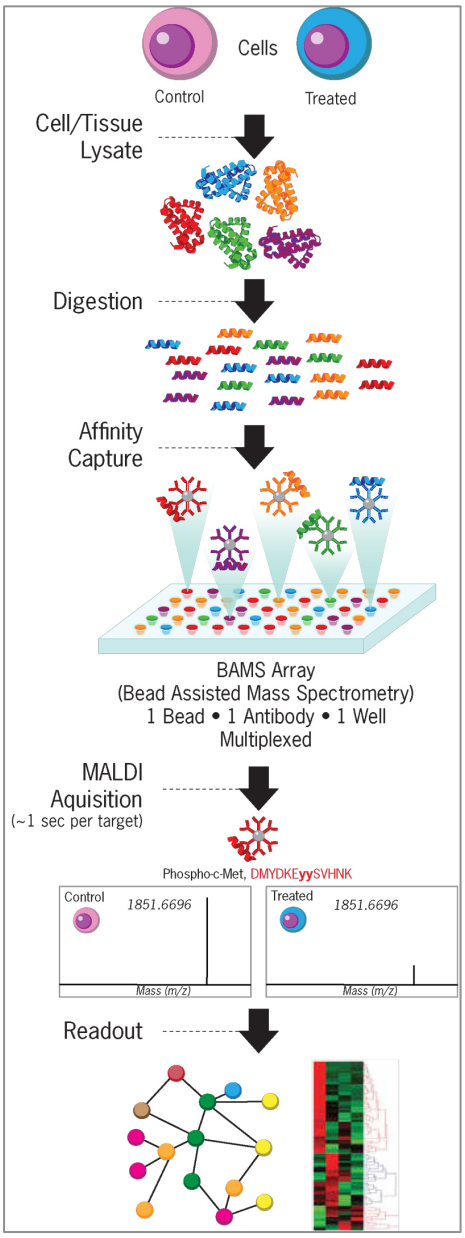

Figure 1. Overall Affi-BAMS workflow. A variety of samples types can be processed for an Affi-BAMS assay (tissue, cell lines, liquid biopsies). The assay consists of the following steps: 1) soluble protein is extracted from the biological material, 2) protein is digested with protease (i.e. trypsin or chymotrypsin), 3) BAMS affinity capture beads are used to enrich for corresponding target peptides to each target protein or post-translational modification, 4) affinity capture beads are washed and then assembled into an ordered array onto an ITO coated MALDI slide, 5) captured peptides are eluted from the beads within the microwell chamber and deposited onto the microarray slide for MALDI MS scanning, 6) the acquired MALDI MS mass spectrum is compared to a spectral library for identification of the target protein or PTM, 7) the MALDI MS intensity signal is used to determine relative quantitation based on an internal reference standard. Pathway profiling is conducted by assembling mixtures of Affi-BAMS beads targeted to proteins within specific signaling networks. BAMS assays are a novel immuno-affinity assay platform for targeted protein analysis. BAMS assays have been configured to monitor key proteins in a wide variety of cellular signaling networks (see www.adeptrix.com/products).

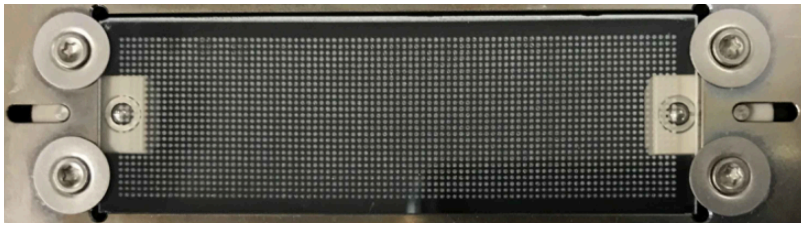

Figure 2. Eluted peptides deposited onto an ITO coated glass slide. Immuno-affinity captured peptides are eluted into an organized array on an ITO coated glass slide for MALDI MS data acquisition. The array contains 2286 spots. MS acquisition takes approximately 1 sec/spot (~ 1.5 hours for entire slide).

Protein Name	Site	Tryptic Peptide Sequence	MH+ (trypsin)	Chymotryptic Peptide Sequence	MH+ (chymotrypsin)
Akt1	T308	(K) DGATMK**t**FCGTPEYLAPEVLEDNDYGR (A)	3131.32	(F) GLCKEGIKDGATMK**t**F (C)	1837.06
Akt2	T309	(K) EGISDGATMK**t**FCGTPEYLAPEVLEDNDYGR (A)	3517.73	(L) CKEGISDGATMK**t**F (C)	1624.67
Akt3	T305	(K) EGITDAATMK**t**FCGTPEYLAPEVLEDNDYGR (A)	3545.78	(L) CKEGITDAATMK**t**F (C)	1653.81
Akt1	S473	(R) RPHFPQF**s**YSASGTA (-)	1733.77	(F) TAQMITITPPDQDDSMECVDSERRPHFPQF**s**Y (S)	3881.17
Akt2	S474	(R) THFPQF**s**YSASIR (E)	1621.68	(Y) DSLGILELDQRTHFPQF**s**Y (S)	2347.48
Akt3	S472	(R) RPHFPQF**s**YSASGR (E)	1717.78	(Y) DEDGMDCMDNERRPHFPQF**s**Y (S)	2727.82
Erk1	T202 & Y204	(R) IADPEHDHTGFL**t**E**y**VATR (W)	2333.39	(F) L**t**E**y**VATRW (Y)	1299.24
Erk2	T185 & Y187	(R) VADPDHDHTGFL**t**E**y**VATR (W)	2305.24	(F) L**t**E**y**VATRW (Y)	1299.24
Gsk3A	S21	(R) TS**s**FAEPGGGGGGGGGGPGGSASGPGGTGGGK (A)	2514.44	(-) MSGGGPSGGGPGGSGRARTS**s**F (A)	2005.03
Gsk3B	S9	(R) TT**s**FAESCKPVQQPSAFGSMK (V)	2369.57	(-) MSGRPRTT**s**F (A)	1220.28
Stat5A	Y694	(K) AVDG**y**VKPQIK (Q)	1298.41	(Y) TPVLAKAVDG**y**VKPQIKQVVPEF (V)	2607.99
Stat5B	Y699	(K) AVDG**y**VKPQIK (Q)	1298.41	(Y) TPVPCESATAKAVDG**y**VKPQIKQVVPEF (V)	3140.53

Table 1. Predicted tryptic and chymotryptic peptides to protein isoforms and their conserved phosphorylation sites. Affi-BAMS assays have been configured for the following proteins: AKT, ERK, GSK3 & STAT5. These proteins exist in multiple isoforms, but can be distinguished using mass spectrometry because the corresponding proteolytic peptides (i.e. trypsin and/or chymotrypsin) have distinct masses that can be used to uniquely identify each protein isoform. The MH+ values correspond to the calculated average mass of the phosphorylated peptide (amino acid residue in lower case bold red text). The site designation for each phosphorylated peptide is annotated in the Site column above for each protein.

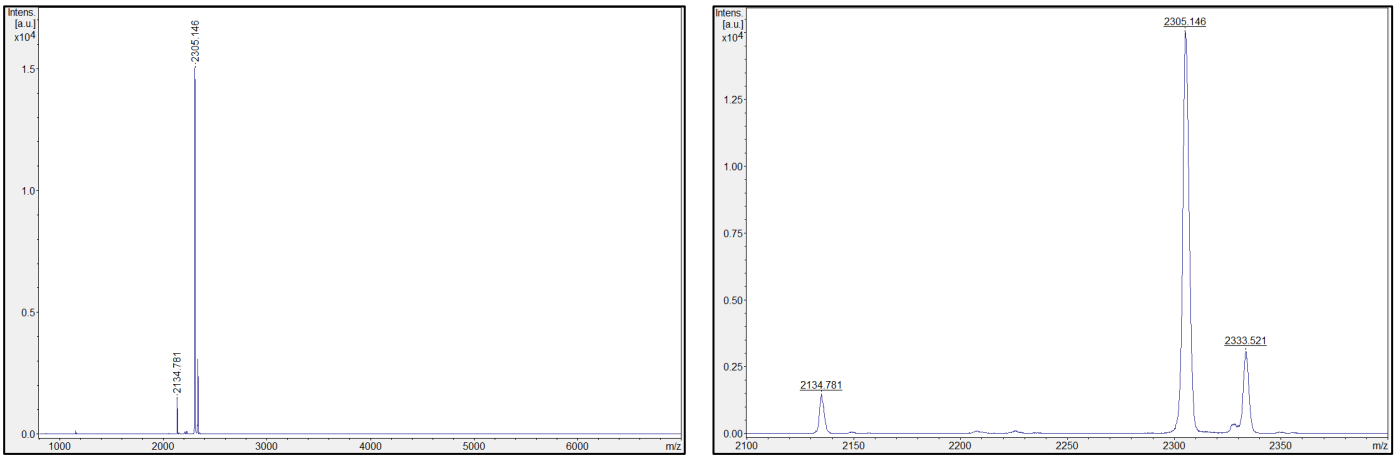

Figure 3. MALDI MS spectrum of the dually phosphorylated tEy peptides from Erk1/Erk2 protein isoforms. The MALDI MS spectrum (linear mode MS) is shown from a BAMS assay using the Affi-BAMS affinity capture bead for Erk1 (T202 & Y204) and Erk2 (T185 & Y187). The target phosphorylated peptides were captured using 200 µg of total protein (trypsin digested) from human gastric cancer cells (MKN45). Both peptides are captured due to the conserved amino acid sequence of the two protein isoforms and the cross reactivity of the validated, rabbit monoclonal antibody. The phosphopeptides obtained from each protein isoform can be distinguished by MALDI MS spectrum. The full scan mass spectrum shows low background and high signal/noise, where approximately 90% of the observed intensity is accounted for by the target peptides (left panel). Looking at the mass range from 2100 m/z – 2400 m/z, you can clearly see well resolved mass measurements for both Erk1 and Erk2 phosphopeptides (right panel). The Erk1 tryptic peptide sequence, IADPEHDHTGFL**t**E**y**VATR, has a calculated average mass of 2333.39 m/z (measured mass 2333.52 m/z). The Erk2 tryptic peptide sequence, VADPDHDHTGFL**t**E**y**VATR, has a calculated average mass of 2305.24 m/z (measured mass 2305.15 m/z). The Affi-BAMS assay for Erk1 (T202 & Y204)/Erk2 (T185 & Y187) is used to perform a multiplex BAMS assay within a single microwell spot.

OVERVIEW

The Affi-BAMS assay platform has been engineered to enable targeted proteomics on existing MALDI MS instrumentation by providing efficient enrichment of target analytes that are spatially localized for rapid screening. The multiplexing capability of the assay allows for analysis of specific signaling networks to advance translational research efforts. Affi-BAMS assays have been configured to monitor many different signaling pathways and areas of disease biology to facilitate basic research and drug development (see www.adeptrix.com/products).