An Affinity Capture MALDI TOF MS Method for High Density Multiplexed Profiling of Total and PTM Protein Biomarker Panels

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INTRODUCTION

Proteomic studies that monitor protein and PTM abundance often employ multi-dimensional analytical methods such as nano-LC-ESI-MS/MS to simplify the inherent sample complexity and wide dynamic range of target molecules within a typical biological specimen. The sophistication of these analytical platforms can inhibit the technology adoption in programs for drug development or biomarker screening due to throughput limitations, data management thresholds and informatics processing needs, required to adequately support these efforts. In this study, we present a method that integrates immunoaffinity purification (IAP) with MALDI TOF MS, which is capable of monitoring hundreds of target analytes in a microarray format, with as little as 5 ug of total protein, in a fraction of the time it takes for one LC-MS/MS acquisition.

METHODS

Cell Culture: MKN-45 and Hela cells were prepared in RPMI media with 10% fetal bovine serum (FBS) and 1X Pen-Strep (Sigma, #P4333) to 75% confluency at 37 °C with 5% CO_2. Prior to chemical treatment, cells were serum starved in RPMI media with 0.2% FBS and 1X Pen-Strep for 12 hrs. SU11274 and staurosporine were used at a final concentration of 1 μM and 0.2 μM, respectively in 0.05% DMSO. Hydrogen peroxide (H_2O_2) was used at a final concentration of 2 mM with a 30 m pre-treatment of 0.1 mM sodium orthovanadate.

Western Blot Analysis: Protein concentrations for lysate supernatants were determined by Bradford assay using Coomassie Plus Protein Assay Reagent (Life Technologies, Carlsbad, CA, #23236), and protein amounts were normalized between samples. Samples were mixed with Laemmli sample buffer (BioRad, #1610747) and run on 4-15% Mini-Protean TGX precast gels (BioRad, #4561083). Proteins were transferred to mini nitrocellulose membranes (BioRad, #1704158) and blocked for 1 h at room temperature in 5% nonfat dry milk (Carnation) in TBS plus 0.1% Tween-20 (TBS-T). Primary antibodies were incubated in 5% BSA in TBS-T overnight at 4°C. Membranes were washed 3 times with TBS-T, incubated with anti-rabbit secondary antibody conjugated with HRP (Cell Signaling Technology) for 1 h at room temperature in 5% milk TBS-T, washed 3 times with TBS-T and developed on the BioRad ChemiDoc Touch Gel Imaging System. Clarity Western ECL blotting substrate (BioRad, #1705060) was used as substrate for blot development.

Preparation of Protein Lysates and Digested Peptides: Cells were washed twice with cold PBS. PBS was removed and cells were scraped in Urea Lysis Buffer (9 M sequanal grade Urea, 20 mM HEPES pH 8.0, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 2.5 mM sodium pyrophosphate). Cells were sonicated 3 times for 20 s each at 15 W output power with a 1-minute cooling on ice between each burst. Sonicated lysates were centrifuged 15 min at 4 °C at 20,000× g. An aliquot of each supernatant was reserved for Western blotting and stored at −80 °C. Supernatants were collected and reduced with 4.5 mM DTT for 30 min at 55 °C. Reduced lysates were alkylated with 10mM iodoacetamide for 15 min at room temperature in the dark. Samples were diluted 1:4 with 20 mM HEPES pH 8.0 and digested overnight with 10 ug/mL trypsin-TPCK (Worthington, Lakewood, NJ, USA, #LS003740) in 1 mM HCl. Digested peptide lysates were acidified with 1% TFA and peptides were desalted over 360 mg SEP PAK Classic C18 columns (Waters, Richmond, VA, USA, #WAT051910). Peptides were eluted with 40% acetonitrile in 0.1% TFA, dried under vacuum, and stored at −80 °C.

Immunoaffinity Enrichment & MALDI Analysis: Protein A/G beads were prepared using NHS-activated XL magnetic agarose beads (400 micron, Cube Biotech) with Protein A/G (Abcam, 1mg/ml) in PBS buffer. Antibodies (2 ug) were conjugated to 5 uL slurry of Protein A/G beads by overnight incubation in PBS with 0.1% BSA. Unbound antibody was removed with three 400 ul washes of PBS with 0.1% BSA. Individual target peptide enrichment was performed using 40 – 1000 ug of purified peptides with 1 – 5 beads. Multiplex target peptide enrichment was performed using 40 – 1000 ug of purified peptides with 1 – 5 beads/target. Peptides were incubated overnight at 4 °C. Beads were washed three times in PBS to remove non specific bound peptides. Remove all liquid from last wash and add 1.5 – 2.0 uL of matrix (10 mg/mL CHCA in 50% ethanol/water, 0.1% formic acid) to elute bound peptide(s) for 15 min at 25 °C. Spot 1uL of eluted peptides (in matrix) onto the MALDI plate. Allow to dry completely before MS analysis using a MALDI TOF instrument (Autoflex Speed, Bruker & SimulTOF ONE, SimulTOF).

METHODS & RESULTS

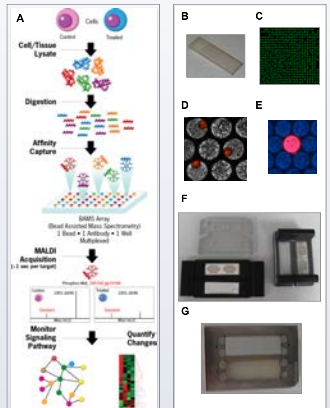


Figure 1. Bead Assisted Mass Spectrometry (BAMS) method and apparatus for affinity enrichment and MALDI MS measurement of target protein-sites. (A) BAMS workflow, (B) picoplate (500 μm wells), (C) arrayed beads (with GFP label), (D) texas red labeled peptide bound to bead, (E) eluted peptide (texas red) in single picoplate well with CHCA matrix (DAPI) resulting in PINK overlay, (F) picoplate bead loader and (G) MALDI plate adapter.

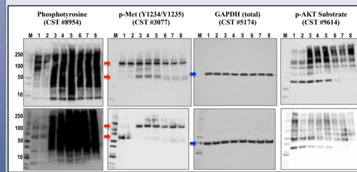


Figure 2. Hydrogen peroxide time course treatment. Western blot of MKN-45 (top) and Hela (bottom) cells. Time course includes serum starved (lane 1), pervanadate treatment at 30 m (lane 2) and peroxide treatment at 5 m (lane 3), 15 m (lane 4), 30 m (lane 5), 60 m (lane 6), 90 m (lane 7) & 120 m (lane 8). Phospho-Met (Y1234/Y1235) is highlighted with red arrow and GAPDH (total) is indicated with blue arrow.

RESULTS

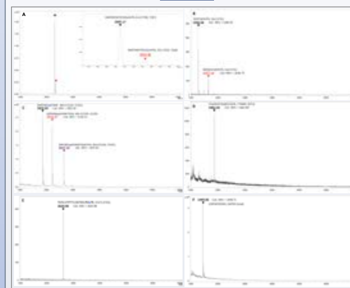


Figure 3. Single Bead Affinity Capture Validation. Target peptides were captured from purified MKN-45 tryptic cell digests on magnetic agarose beads, and eluted from a single 400 micron bead for MALDI TOF MS analysis. MS spectra (2000 summed MALDI spectra) of enriched peptides are shown using the following antibodies: (A) p-ERK1/2 (T202/Y204 & T185/Y187) CST# 4370, (B) p-Cdc2 (Y15) CST# 4539, (C) Met (Y1234, Y1235) CST# 3077, (D) CTNNB1 (S552) CST# 5651, (E) Stat-3 (Y705) CST# 9145 & (F) GAPDH (total) CST# 5774.

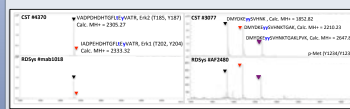


Figure 4. Comparison of p-Erk1/2 and p-Met antibodies from CST and R&D Systems. Antibodies from CST and R&D Systems for both p-Erk1/2 (Left) and p-Met (Right), with the following modifications, p-Erk1/2 (Y185/Y187, Y202/Y204) & p-Met (Y1234/Y1235), were tested for single bead enrichment of target peptide.

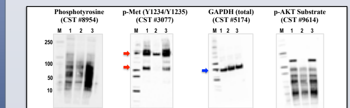


Figure 5. SU11274 and Staurosporine drug treatment of MKN-45 cells. Western blot of MKN-45 treated with DMSO (lane 1), SU11274 (lane 2) and Staurosporine (lane 3). Phospho-Met (Y1234/Y1235) is highlighted with red arrows and GAPDH (total) is indicated with blue arrow.

RESULTS

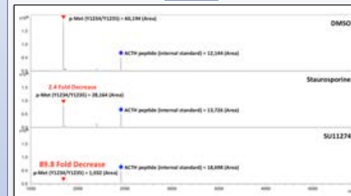


Figure 6. Single-bead enrichment and MALDI analysis for p-Met (Y1234/Y1235) in drug treated MKN-45 cells. The relative abundance of the activation loop phosphotyrosine sites of Met were measured using BAMS. An internal standard peptide (ACTH peptide, MH+ 2466.68) was included in the matrix at a fixed quantity (~ 500 fmoles). The relative abundance of p-Met (Y1234/Y1235) site was measured from the DMYDKEyySVHNK peptide (1852.82 MH+) to show a **2.4- and 89.8-fold reduction** upon staurosporine & SU11274 treatment, respectively. These results are consistent with the western blot results and previous reports based on LC-MS/MS analysis.[4]

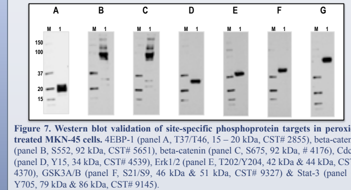
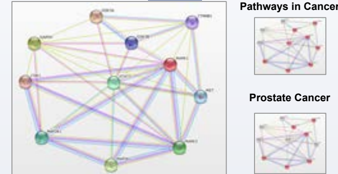


Figure 7. Western blot validation of site-specific phosphoprotein targets in peroxide treated MKN-45 cells. 4EBP-1 (panel A, T37/T46, 15 – 20 kDa, CST# 2855), beta-catenin (panel B, S552, 92 kDa, CST# 5651), beta-catenin (panel C, S675, 92 kDa, # 4176), Cdc-2 (panel D, Y15, 34 kDa, CST# 4539), Erk1/2 (panel E, T202/Y204, 42 kDa & 44 kDa, CST# 4370), GSK3A/B (panel F, S21/S9, 46 kDa & 51 kDa, CST# 9327) & Stat-3 (panel G, Y705, 79 kDa & 86 kDa, CST# 9145).

[illegible]

Figure 8. Validation of Single Bead Capture & MALDI Detection of Target Peptides. The following proteins and corresponding peptides have been validated by MALDI TOF MS, using antibodies for the specified proteins and site-specific phospho-peptides along with peptides from tryptic digests of MKN-45 cells treated with hydrogen peroxide.

RESULTS



Pathways in Cancer

Prostate Cancer

Figure 9. STRING Protein interaction network diagram of validated protein-site targets. An array of antibodies can be customized to probe specific cellular signaling networks and utilized as a screening tool for a variety of research and development efforts. The antibody array can include a combination antibodies directed to site-specific PTMs (e.g. phospho-, acetyl-, methyl-, succinyl-) and to the total protein. The list of validated antibodies characterized in this study include markers for signaling networks in cancer development including prostate cancer.

CONCLUSIONS

- The BAMS assay can be configured to efficiently monitor tens to hundreds of protein biomarkers from a variety of biological samples.
- The binding capacity of a single bead is sufficient to measure the abundance of a target protein-site with high signal-to-noise from as little as 5 ug of total protein extract.
- MALDI MS data acquisition of the BAMS assay enables one to rapidly survey key protein-site targets within critical signaling nodes.
- The bottom-up approach accommodates multiple proteases to expand the number of distinct peptides per target protein, improve the specificity for the biomarker and extend the panel of qualified antibody reagents
- Standard methods for both MS- and MS/MS-based quantitation can be implemented with the BAMS assay for high content screening applications

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