

Total Protein Profiling Employing a Novel Protein Fraction Method Combined with Tandem Mass Tag Labeling

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INTRODUCTION

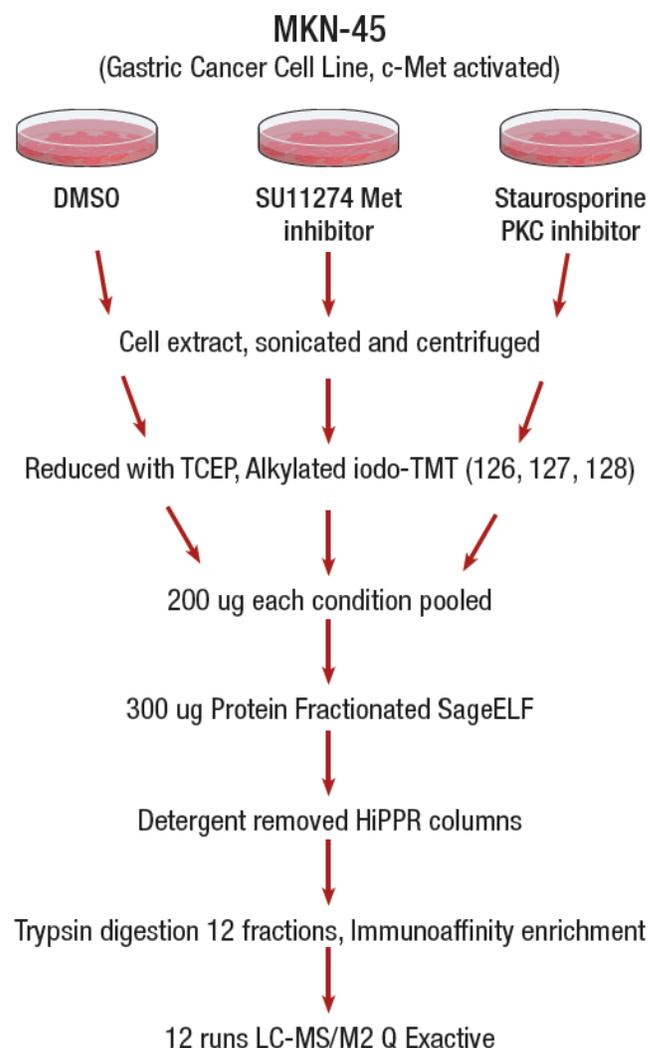
Accurate and reproducible quantitation of cellular proteins is a critical component to the analysis of cellular physiology and crucial to the interpretation of significant alterations of post-translationally modified proteins. A number of established strategies exist to determine the absolute and relative quantities of peptides and their parent proteins from complex biological sources, including iTRAQ[®], TMT[™], SILAC, ICAT[™], 2DE, DIGE, and label-free methods. In this study, we evaluate how fractionation at the protein level can impact the accuracy of protein quantitation using protein-level fractionation in conjunction with tandem mass tag methods on human gastric carcinoma cell line, MKN-45, in response to the Met inhibitor, SU11274, or the PKC inhibitor, staurosporine.

METHODS

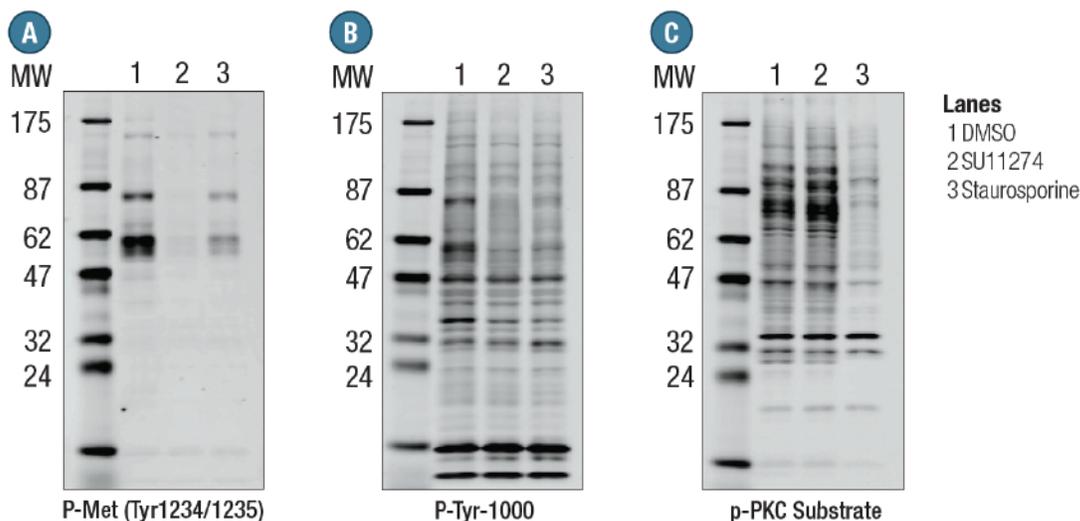
MKN-45 cells were treated with either DMSO or one of the kinase inhibitors, SU11274 or staurosporine, for 2 hours. 200 µg of total soluble protein from each sample condition were labeled on cysteine with the isobaric Tandem Mass Tags[™] (TMT[™]) reagent (Thermo Scientific[™]). Equal amounts of labeled total protein from each condition were pooled, and the proteins were separated into 12 size exclusion fractions by agarose gel-SDS electrophoresis using a SageELF[™] (Sage Science). Residual SDS was removed using HiPPR[™] resin (Thermo Scientific[™]) and proteins were then digested with trypsin. The cysteine-TMT labeled peptides were immunoaffinity purified prior to LC-MS/MS analysis using a Q Exactive[™] mass spectrometer (Thermo Scientific[™]). Data was acquired using a top10 DDA method. MS/MS spectra were assigned using SEQUEST[®] (University of Washington), and quantification was performed using the relative ratios of the corresponding TMT marker ions.

EXPERIMENTAL DESIGN

Cells were treated with either DMSO, SU11274, or Staurosporine, and cellular extracts were then labeled with the isobaric mass tags in channels 126, 127, 128, respectively. Extracts were concentrated to ~10 mg/ml then pooled and loaded on a SageELF. The SDS was removed from proteins in each fraction, samples were digested with trypsin, immunoaffinity enriched, desalted and run on an Orbitrap Q-Exactive, and finally proteins were identified using SEQUEST.

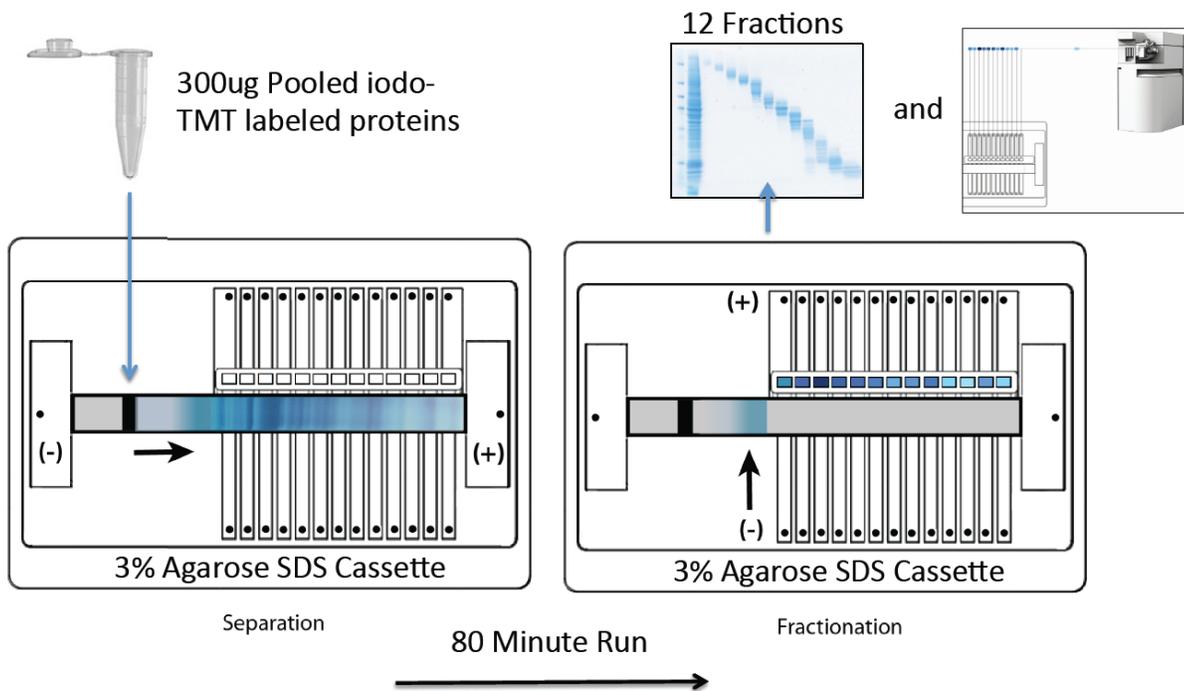


CONTROL WESTERN BLOTS



Western blot analysis of extracts from MKN-45 cells (20 µg of protein per condition) treated with either DMSO, SU11274, or Staurosporine, using Phospho-Met (Tyr1234/1235) (3D7) Rabbit mAb #3129 (A), Phospho-Tyrosine (P-Tyr-1000) Rabbit mAb #8954 (B), or Phospho-(Ser) PKC Substrate (P-S3-101) Rabbit mAb #6967 (C).

FRACTIONATION OF CYSTEINE LABELED PROTEINS



300 µg of pooled proteins from the three experimental conditions (DMSO, SU11274, and Staurosporine) were labeled on cysteine residues and subjected to size based fraction and pre-programmed elution into 12 fractions using the SageELF. Following reduction and mass tag labeling, the extracts were pooled, loading buffer was added and samples were heated to 85°C for 6 minutes. Following elution, ~60% of each fraction was run per lane on a 4–20% SDS PAGE gel.

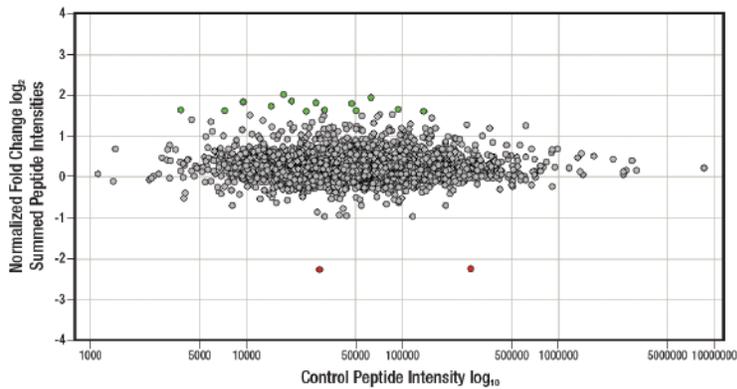
SUMMARY OF TANDEM LC-MS/MS RESULTS

Raw mass spectrometry data was searched against human UniProt/Swis-Prot database using SEQUEST, FDR was 1%.

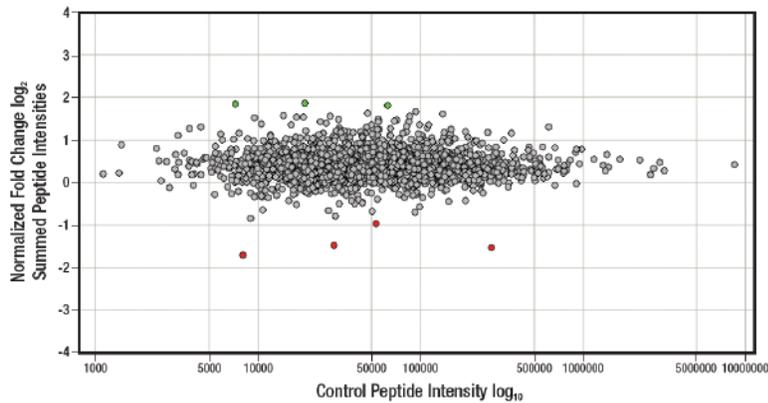
FRACTION #	TMT PEPTIDES		PROTEINS
	REDUNDANT	NON-REDUNDANT	
1	5,373	2,773	266
2	5,465	2,903	326
3	3,768	2,074	317
4	3,278	1,728	370
5	661	362	34
6	1,675	968	209
7	2,098	1,108	227
8	1,832	994	157
9	1,828	1,007	126
10	4,593	2,232	625
11	3,430	1,678	283
12	1,840	1,024	98
Combined Results	35,841	18,851	3,038

SCATTER PLOTS OF FOLD CHANGE RATIOS

A Scatter Plot DMSO Control: SU11274

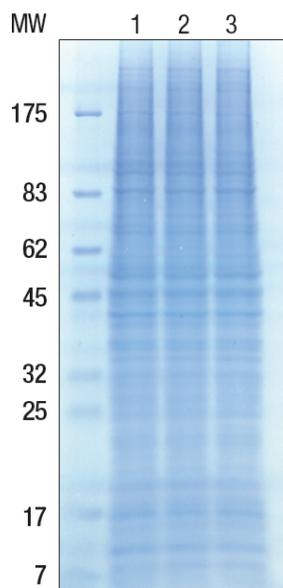


B Scatter Plot DMSO Control: Staurosporine



The summed peptide intensities for 3,038 parent proteins; median log₂ normalized ratios were produced for DMSO control vs. SU11274 (A), and DMSO control vs. Staurosporine (B). Fold change greater than 2.5-fold is shown in green, less than 2.5-fold is shown in red.

TOTAL PROTEIN COOMASSIE STAINED GEL

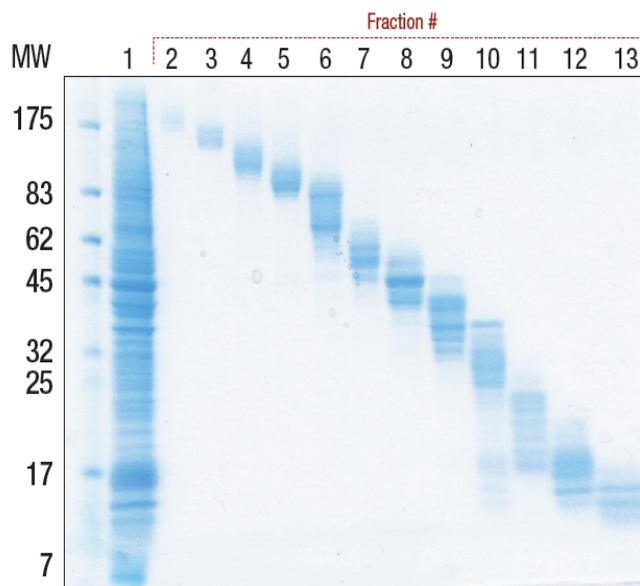


Following 2 hr treatment with either DMSO, SU11274, or Staurosporine, 20 µg of each extract were run on a 4–20% gradient gel and stained with Coomassie® Brilliant Blue.

Lanes

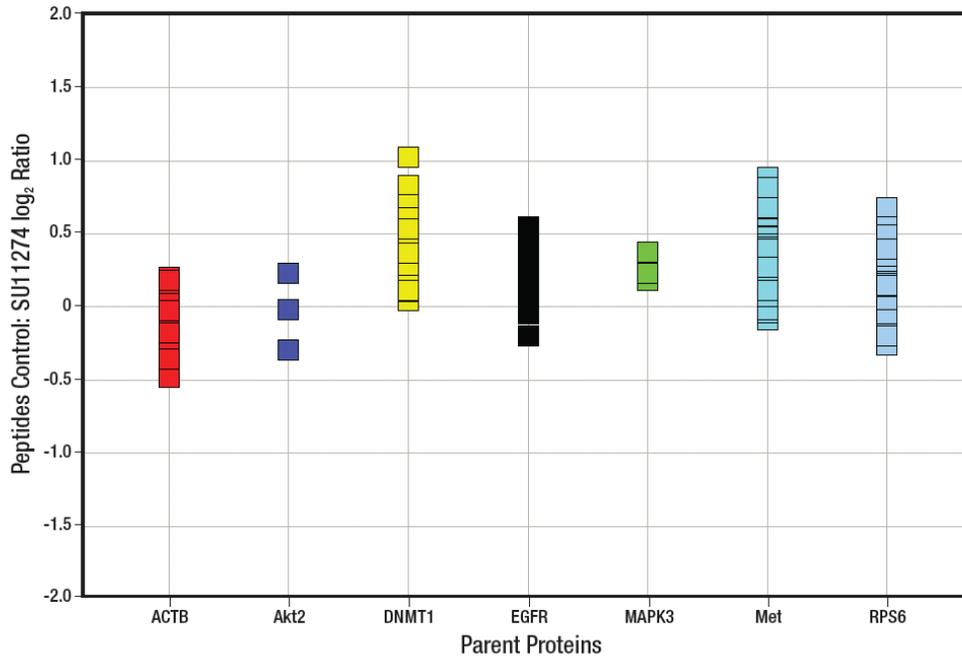
- 1 DMSO
- 21 µM SU11274
- 3200 nM Staurosporine

COOMASSIE STAINED GEL OF MKN-45 CELL EXTRACTS FRACTIONATED ON SAGE ELF

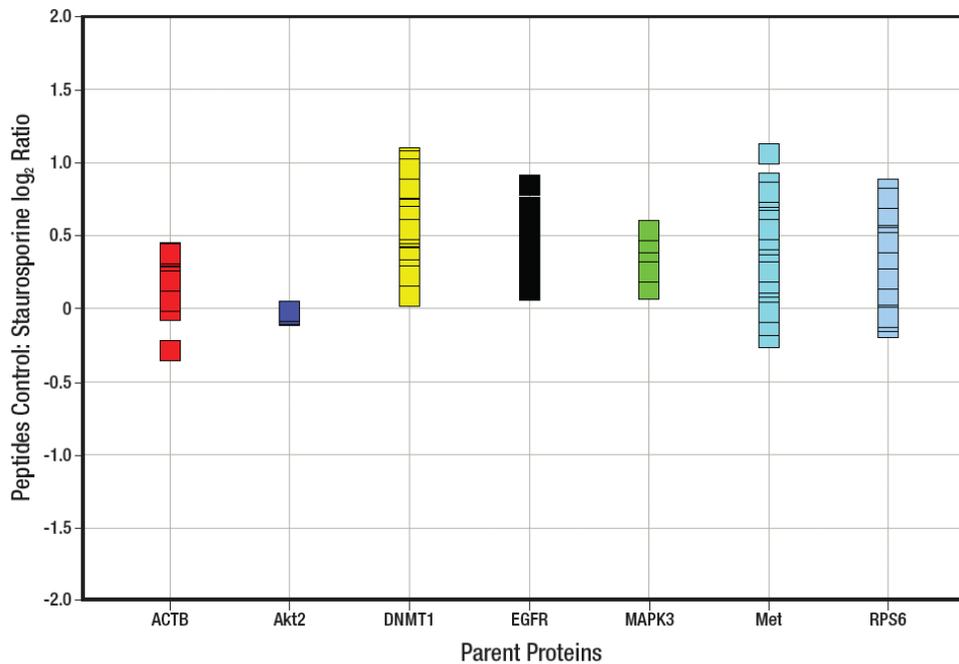


Following iodo-TMT labeling 100 µg of protein from each experiment (DMSO, SU11274, and Staurosporine) were pooled and fractionated on a SageELF, using a 3% agarose SDS cassette for 80 minutes at 100 volts. Following pre-programed elution, 16 µl (of 26 total) of each fraction (lanes 2–13) were loaded on a 4–20% SDS PAGE gradient gel (Invitrogen) then stained with Coomassie Brilliant Blue. Lane 1 is 20ug of original extract pool.

A Peptide Ratios Control: SU11274



B Peptide Ratios Control: Staurosporine



Peptide abundance was compared in the control to treated samples for 7 different proteins. Ratios of median log₂ normalized peptide intensities were compared for peptides from 7 different genes, DMSO control vs. SU11274 (A), and DMSO control vs. Staurosporine (B).

CONCLUSIONS

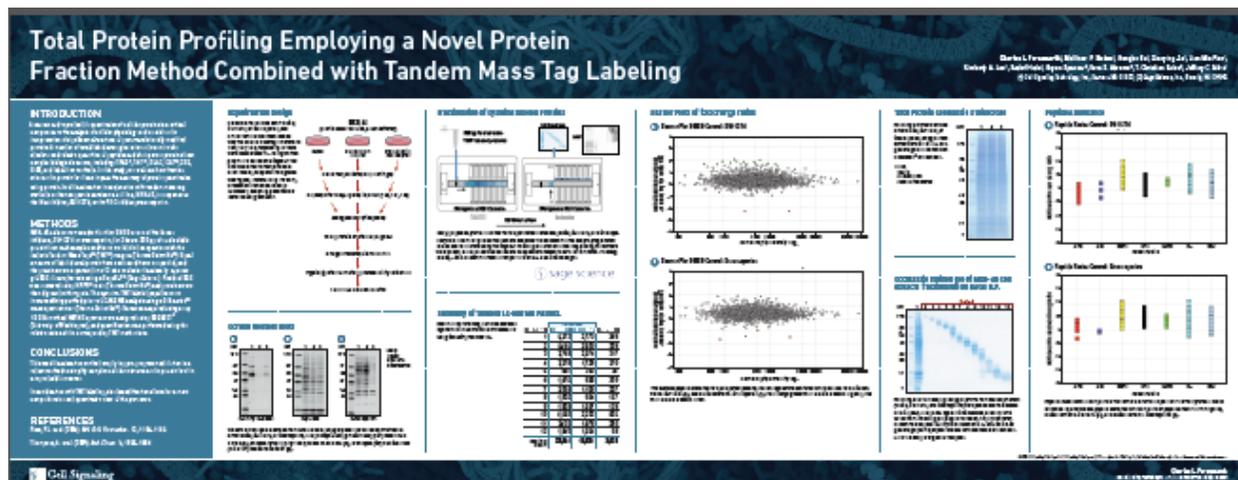
- This novel fractionation method employing pre-programmed elution is a robust method to simplify complex cellular mixtures at the protein level in a reproducible manner.
- In combination with TMT labeling, size based fraction allows for a more comprehensive and quantitative view of the proteome.

REFERENCES

Ross, P.L. et al. (2004) Mol. Cell. Proteomics. 12, 1154–1169.

Thompson, A. et al. (2003) Anal. Chem. 8, 1895–1904.

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