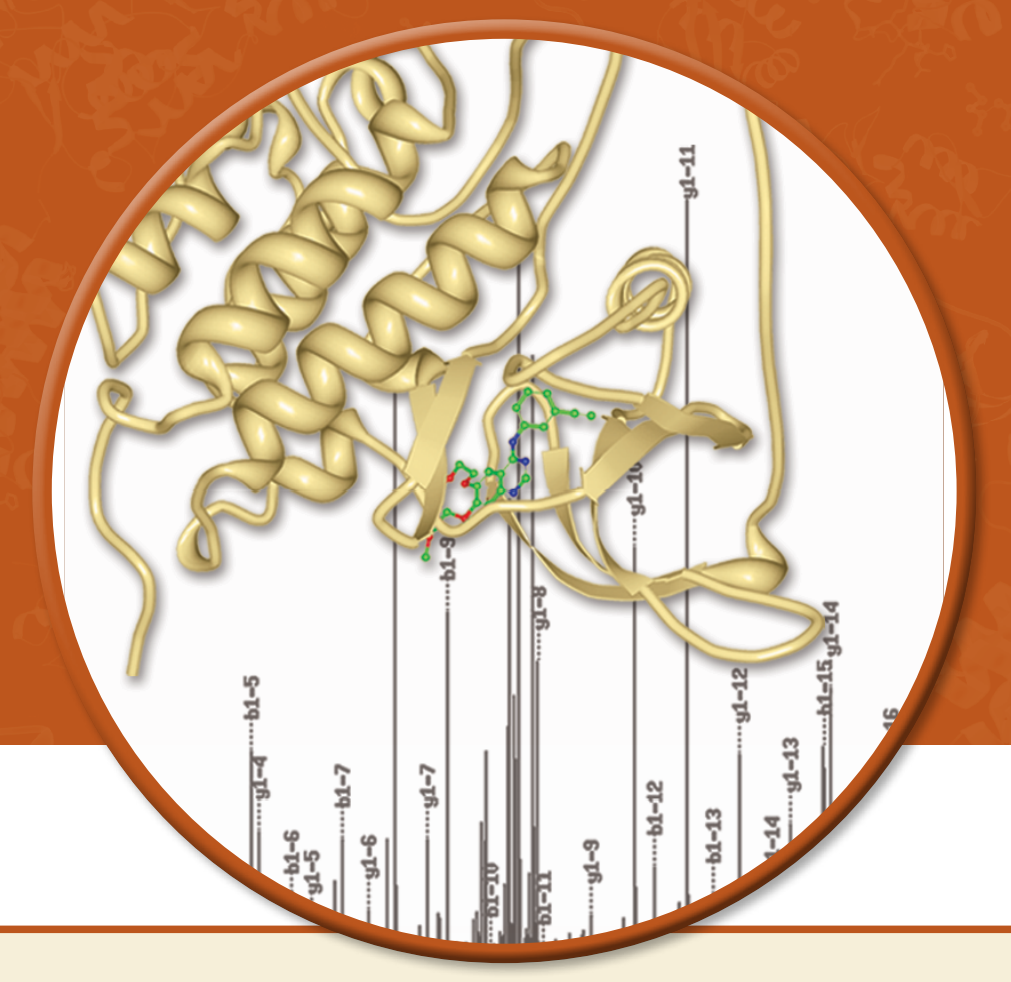


Development and application of highly sensitive SIM and SRM-based phosphopeptide immunoassays using the Q Exactive™ mass spectrometer

Kimberly A. Lee, Joan MacNeill, Jing Zhou, Jian Yu, Matthew P. Stokes, Jeffrey C. Silva, Ailan Guo, Michael J. Comb *Cell Signaling Technology, Danvers MA USA 01923*

Abstract

There is a clinical need to identify active signaling pathways in patient tumors from very small biopsy samples to inform treatment decisions. Mass spectrometry-based assays are desirable and are being more widely developed and implemented for candidate biomarkers due to their exquisite specificity, relatively fast development time, and their ability to be highly multiplexed. These assays have traditionally been performed on triple quadrupole mass spectrometers, but the recent introduction of the Q Exactive™ mass spectrometer has enabled pseudo-SRM assay development with higher resolution and excellent dynamic range. Here we demonstrate high specificity and sensitivity of both Selected Reaction Monitoring (SRM) and Single Ion Monitoring (SIM) assays for peptides and phosphopeptides and apply this method to quantify peptides immunoenriched from biological samples.

All assays were performed using a Q Exactive mass spectrometer coupled with an EASY nLC™ II HPLC through a New Objective nanospray source. Native and isotope-labeled versions of each assayed peptide were synthesized and quantified in house and peptide immunoenrichment was performed in multiplexed fashion, with dozens of site-specific and peptide-specific antibodies mixed to enable a single immunoprecipitation for and subsequent quantification of multiple target peptides and phosphopeptides.

Linear response to input peptide amount was observed over five orders of magnitude using our Q Exactive-based SIM method in both simple and complex background matrices while SRM scans showed linear response over four orders of magnitude in a complex matrix. Comparison of SIM scans to normal scans demonstrated a consistent improvement in limit of detection in all matrices tested, with low amol sensitivity consistently achieved. Instrument settings including gradient length, SIM maximum ion time, and resolution were optimized for sensitivity and specificity.

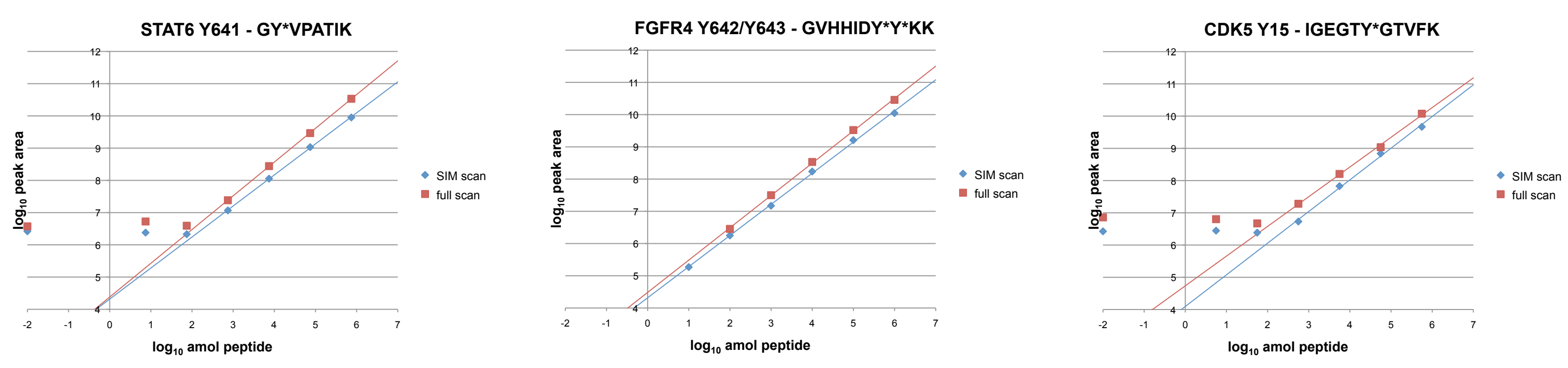
Sensitivity of the immunoenrichment-LC-MS assays was determined using the reversed curve strategy in a background of human lung tissue peptides. Lower limit of quantification values for these assays ranged from 100 amol to 1 fmol input peptide. Application of the SIM method to peptides immunoenriched from the H3255 cell line demonstrated our ability to quantify many phosphopeptides from 5 ug total input protein with 22 peptides multiplexed in a single assay. Xenograft tumor tissue was used to demonstrate a 52-plex assay, with peptides quantified at 400 ug – 4 mg input wet weight tissue equivalent amounts. These sensitivity levels are sufficient to allow quantification of relevant phosphopeptides from core tumor biopsies, potentially informing treatment decisions and determining pharmacodynamic drug responses in clinical trials.

Objectives

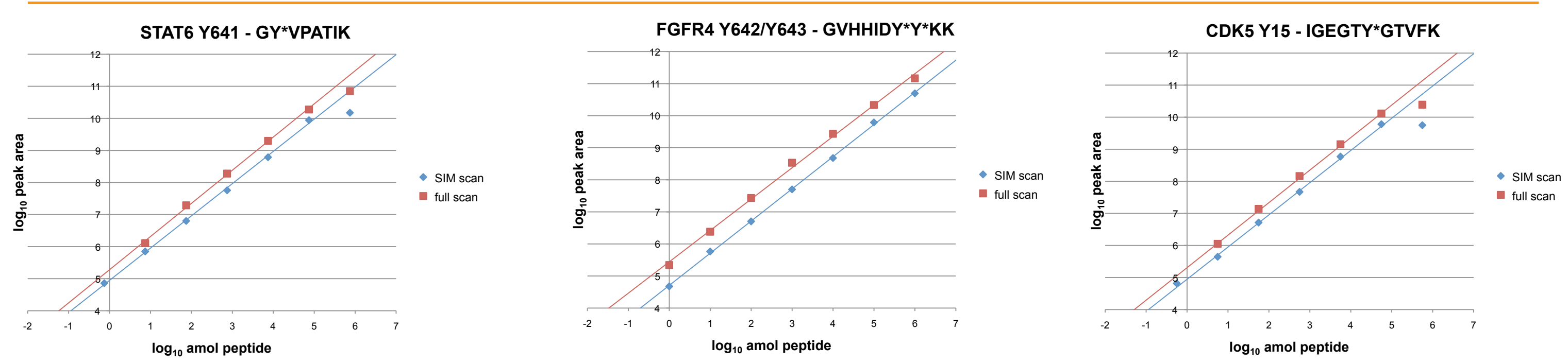
- Establish method for highly sensitive analysis of targeted phosphopeptides enriched from a complex background
- Evaluate and optimize LC-MS assay methods for maximal sensitivity and specificity
- Determine linear range and LLOQ for multiplexed LC-MS immunoassays
- Apply multiplexed method to quantify phosphopeptides from low levels of cell line peptides and xenograft tissue

Establishing linear range of LC-MS system

High Complexity Background



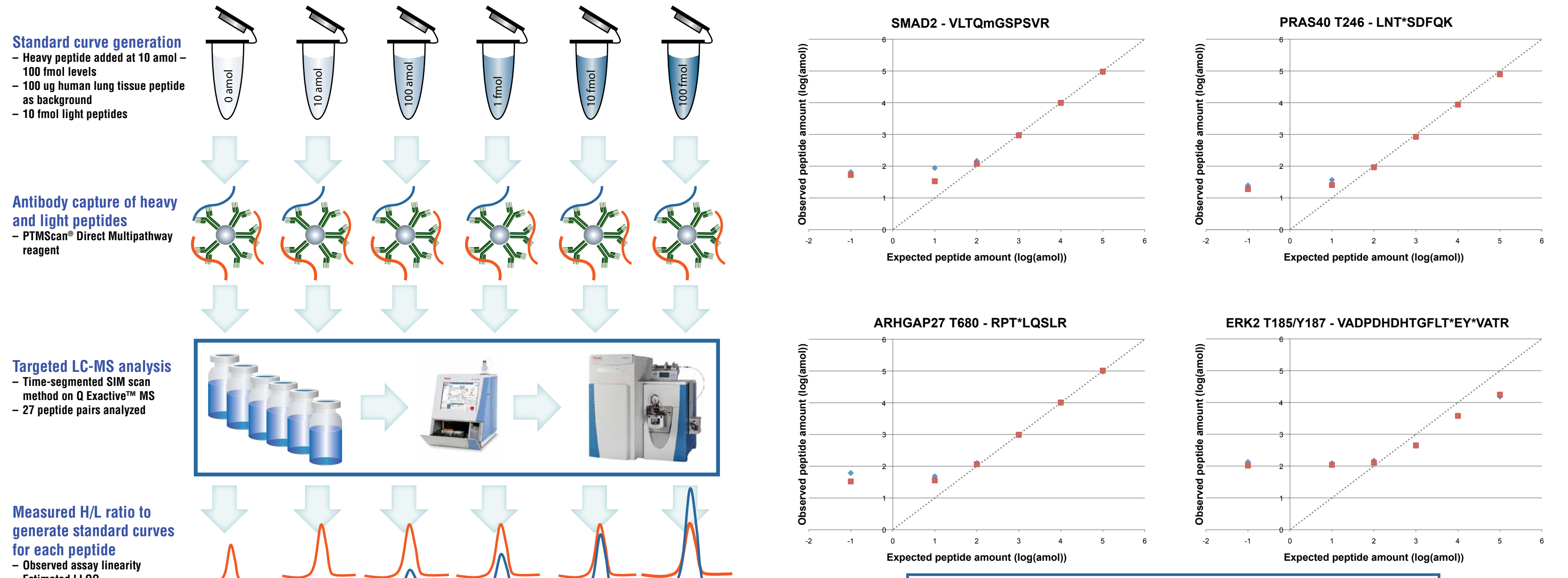
Low Complexity Background



- Q Exactive™ SIM scans confer an additional order of magnitude of sensitivity compared to full scans
- Five orders of magnitude linear range is consistently observed in low complexity background

Figure 1. Serial dilutions of heavy isotope-labeled AQUA™ peptides in a complex background (167 ng tryptic peptides from MKN45 cell line) or a less complex background (50 fmol BSA tryptic digest). Extracted ion chromatograms were generated for each peptide either from full scans (red) or from SIM scans (blue) and integrated peak areas were plotted against the input peptide amount. Although peptide intensities are higher in full scan analyses, the improved signal/noise of SIM scans allows higher overall sensitivity, with detection of peptides at low to sub-amol levels in the linear range. In complex backgrounds, most peptides were not quantifiable at the lowest levels due to background signal, but reducing complexity allowed linear instrument response across at least five orders of magnitude. Data points corresponding to peak areas of samples with no heavy isotope-labeled peptides injected are plotted at log10 amol peptide= -2.

Immunoassay sensitivity determination



Standard curve generation
- Heavy peptide added at 10 amol – 100 fmol levels
- 100 ug human lung tissue peptide as background
- 10 fmol light peptides

Antibody capture of heavy and light peptides
- PTMScan® Direct Multipathway reagent

Targeted LC-MS analysis
- Time-segmented SIM scan method on Q Exactive™ MS
- 27 peptide pairs analyzed

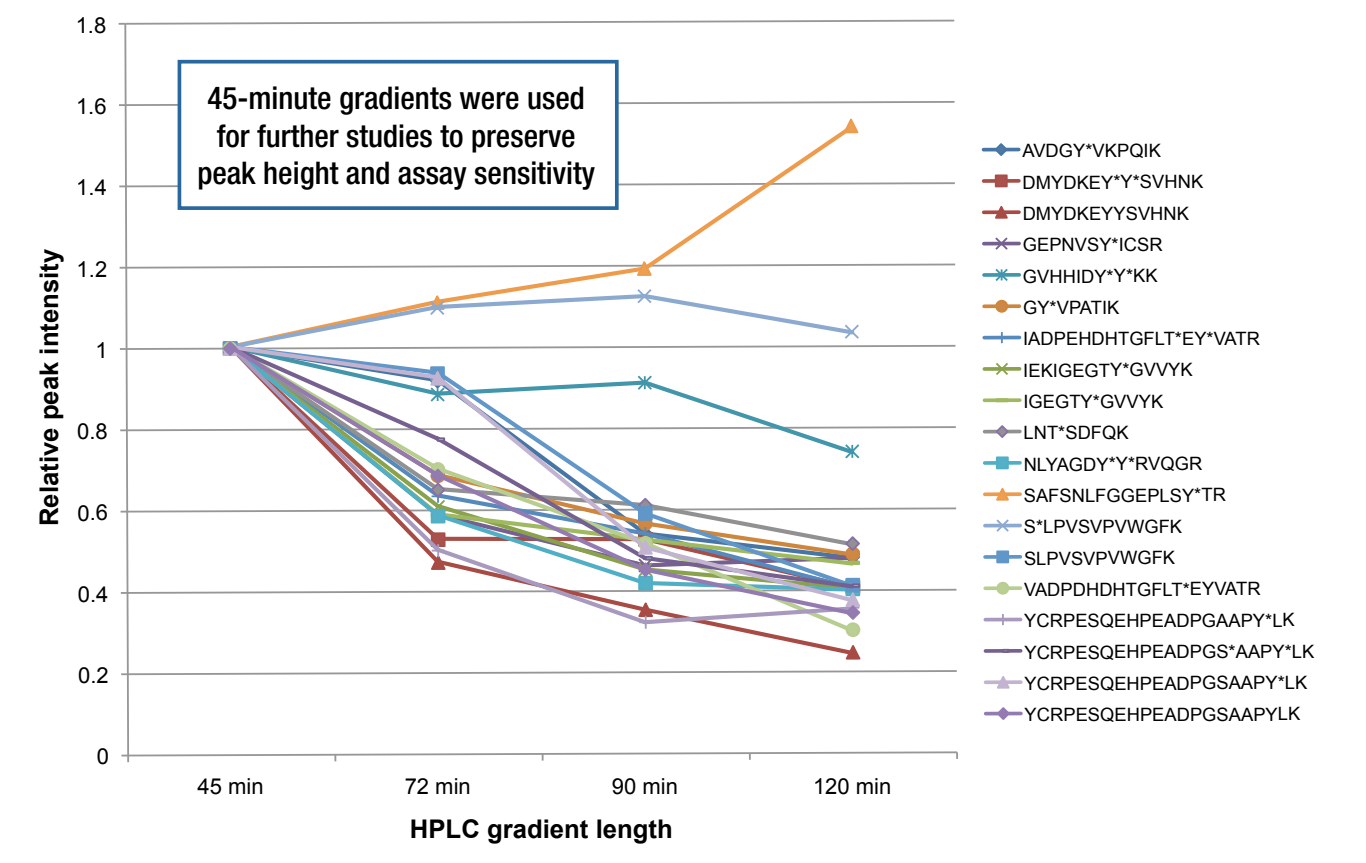
Measured H/L ratio to generate standard curves for each peptide
- Observed assay linearity
- Estimated LLOQ

Many peptide and phosphopeptide LC-MS immunoassays have LLOQ < 100 amol in multiplexed format

Figure 6. Left panel: Experimental details and flowchart. Right panel: Representative data are presented, with observed peptide amount plotted against input peptide amount on a log scale. Data points corresponding to calculated peptide amounts for samples with no heavy isotope-labeled peptides added are plotted at log10 amol peptide= -1. Duplicates of each data point are graphed individually, though significant overlap of measured values is observed. A theoretical x=y dashed line is plotted on each graph to aid in visualization of assay linearity and quantitative assay performance. Data points below the lower limit of quantification appear at the same observed peptide amount as the blank, with all signal derived from sample background. The calculated ERK2 phosphopeptide amounts are in all cases lower than the expected amounts, due to the presence of background native phosphorylated ERK2 peptide in the human lung sample matrix used for this study. To generate these curves we held the input light peptide level constant and varied the heavy peptide (reversed curve strategy). In the case of ERK2, lower right panel, the additional native light peptide in the lung lowers the heavy/light ratio, artificially lowering the calculated amount of heavy ERK2 phosphopeptide.

LC-MS method optimization

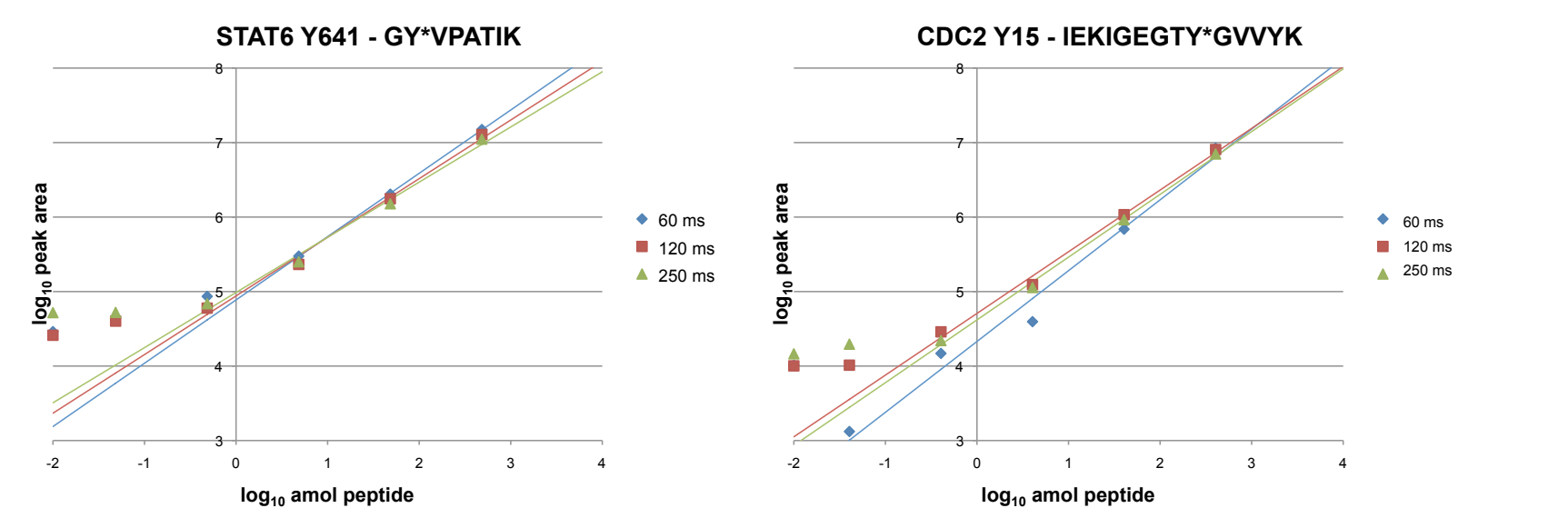
Gradient length evaluation



45-minute gradients were used for further studies to preserve peak height and assay sensitivity

Figure 2. 10 fmol of each peptide was injected in a background of 50 fmol BSA tryptic digest and analyzed with an 8-32% linear CH3CN gradient developed over 45, 72, 90, or 120 min. The peak height for the dominant charge state of each peptide was measured and plotted for each gradient length. For most peptides, a significant decrease in peak height is observed at longer gradient lengths, which is expected to result in decreased sensitivity.

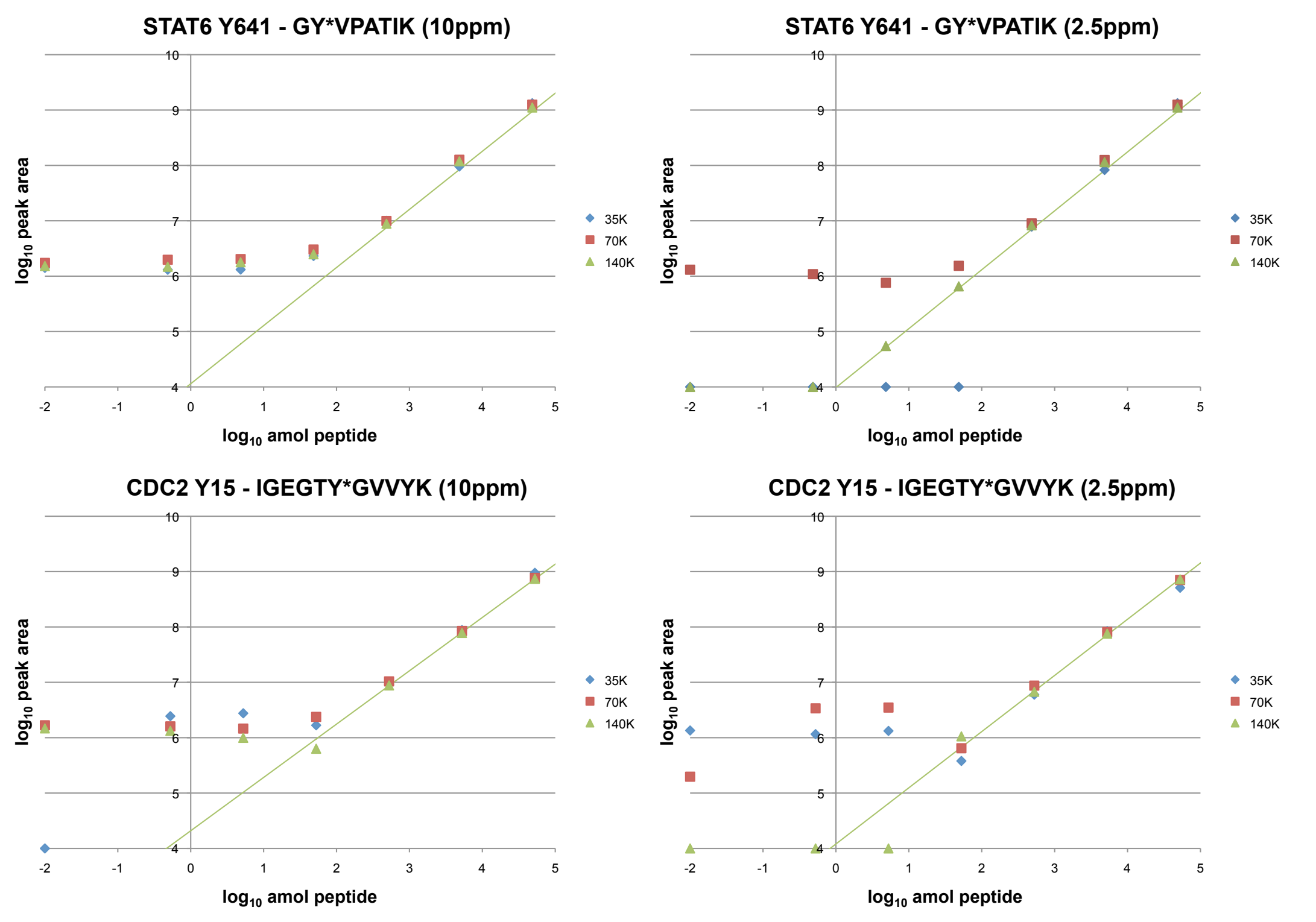
SIM maximum ion time evaluation



120 ms maximum ion time provided greater reproducibility than 60 ms and was selected for further studies

Figure 3. Testing SIM maximum ion time settings. 60 zmol – 600 amol each heavy-labeled peptide was diluted in 50 fmol BSA tryptic digests, and samples were analyzed using targeted SIM scans with maximum SIM ion times of 60, 120, and 250 ms. Peak areas for each of five peptides were integrated and plotted against input peptide amount. Data points corresponding to peak areas of samples with no heavy isotope-labeled peptides injected are plotted at log10 amol peptide=-2. Representative data are presented. Similar results were obtained for each ion time tested, but data for 60 ms method seemed slightly more variable, so 120 ms maximum ion time was selected for further studies.

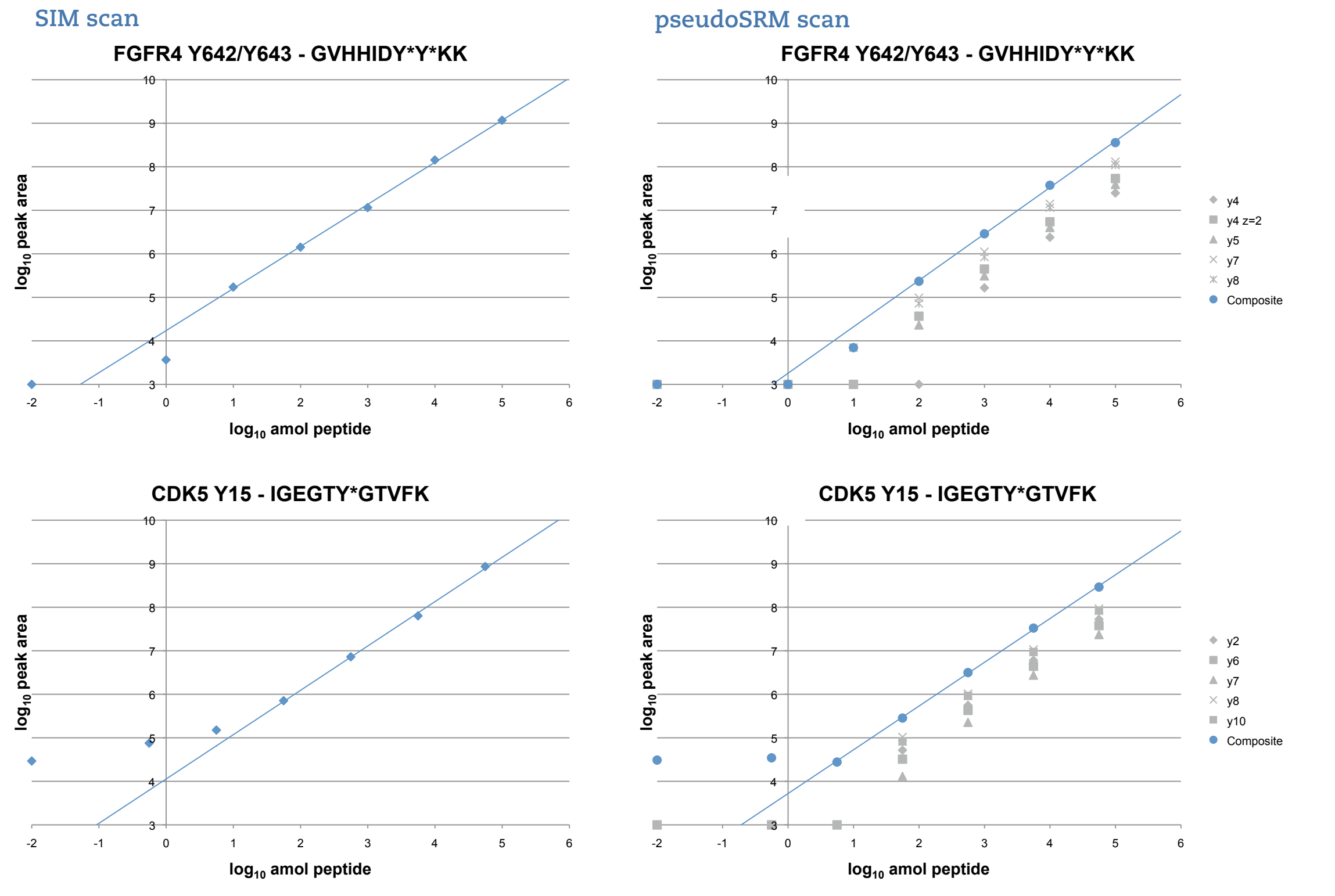
SIM scan resolution evaluation



SIM resolution of 140k provided improved sensitivity through background reduction for some peptides and was selected for further studies

Figure 4. Testing SIM resolution settings. 600 zmol – 60 fmol each heavy-labeled peptide was diluted in 167 ng MKN45 cell line total protein tryptic digests, and samples were analyzed using targeted SIM scans with 35k, 70k, and 140k resolution settings. Extracted ion chromatograms were generated using a mass accuracy of 10 ppm or 2.5 ppm for each of five peptides. Peak areas were integrated and plotted against input peptide amount. Curve fits presented were generated to the linear portion of the 140k resolution data. Data points corresponding to peak areas of samples with no heavy isotope-labeled peptides injected are plotted at log10 amol peptide=-2. Two example peptides are presented. Similar results were obtained for each resolution setting, but background reduction and therefore improved sensitivity could be observed for some peptides when combining the highest resolution setting with a narrow mass window (2.5 ppm) for peak detection. Consequently, 140k was selected as the SIM scan resolution for further studies.

PseudoSRM scan evaluation

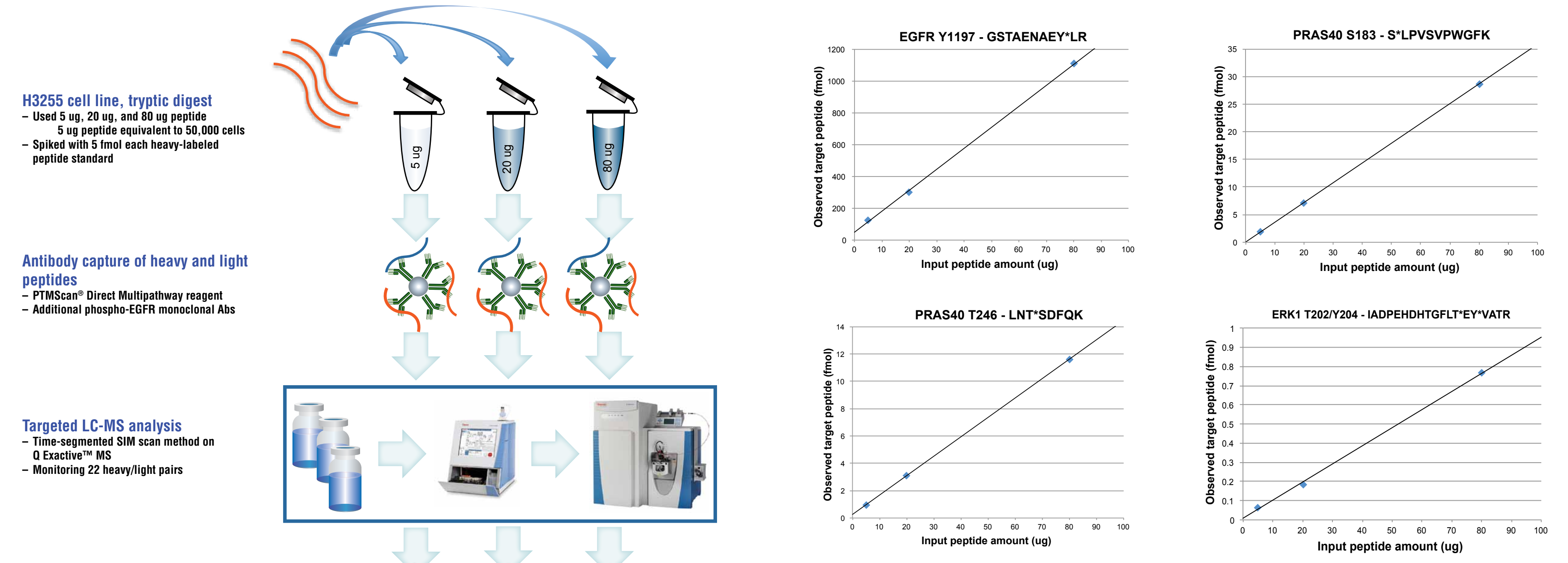


Pseudo-SRM scans did not improve assay performance over the SIM scan method. Any increases in specificity are offset by decreased sensitivity.

Figure 5. Pseudo-SRM (PRM) scan evaluation. 600 zmol – 60 fmol each heavy-labeled peptide was diluted in 167 ng MKN45 cell total protein tryptic digests, and samples were analyzed using either SIM scan method or data-independent MS/MS scans with an HCD NCE setting of 25%. Five daughter ions were selected from each spectrum for peptide quantitation. Extracted ion chromatograms were generated from each of these daughter ions as well as the summed area of all five ions (labeled Composite). Peak areas were integrated and plotted against input peptide amount. Curve fits presented were generated to the linear portion of the SIM data and the composite daughter ion measurements. Data points corresponding to peak areas of samples with no heavy isotope-labeled peptides injected are plotted at log10 amol peptide=-2. Representative data are presented. Background reduction affected by use of PRM scans did not increase sensitivity of the assay as compared to SIM scans, since high resolution SIM scans (140k) were employed. For some peptides, a reduced linear range was observed when using the PRM scan (see FGFR4 peptide above), so SIM scans were selected for application of this method.

Application of multiplexed LC-MS immunoassay

Application of LC-MS immunoassay to cell line



H3255 cell line, tryptic digest
- Used 5 ug, 20 ug, and 80 ug peptide
  5 ug peptide equivalent to 50,000 cells
- Spiked with 5 fmol each heavy-labeled peptide standard

Antibody capture of heavy and light peptides
- PTMScan® Direct Multipathway reagent
- Additional phospho-EGFR monoclonal Abs

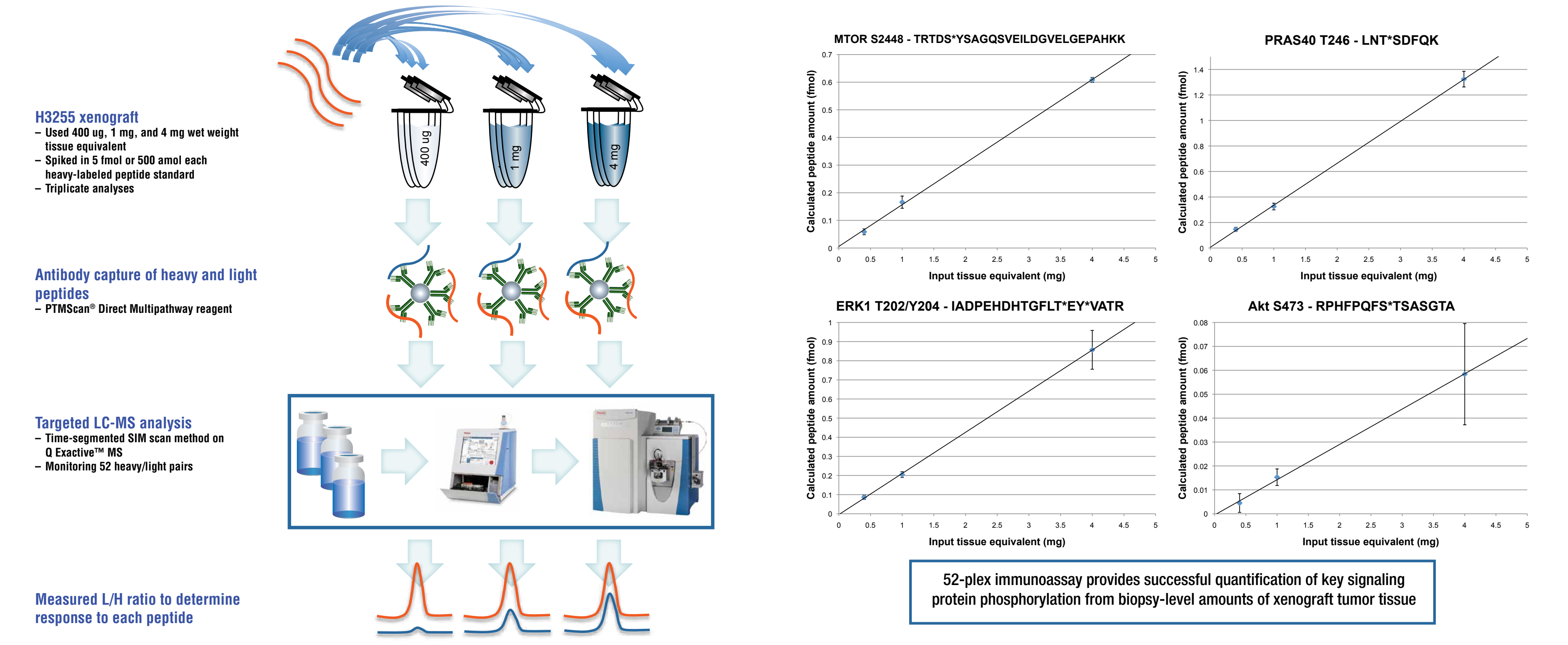
Targeted LC-MS analysis
- Time-segmented SIM scan method on Q Exactive™ MS
- Monitoring 22 heavy/light pairs

Measured L/H ratio to determine response to each peptide

Assay linearity is observed for phosphopeptides with native cell line levels varying across three orders of magnitude

Figure 7. Application of LC-MS immunoassay to H3255 cell line tryptic peptides. Left panel: Experimental details and flowchart. Right panel: Representative data are shown, with observed target peptide amount plotted against input total cell line peptide amount. Linear curve fits for each peptide are presented. A value of 1 fmol observed target peptide at the 5 mg input peptide level is equivalent to 12,000 copies per cell.

Application of LC-MS immunoassay to xenograft



H3255 xenograft
- Used 400 ug, 1 mg, and 4 mg wet weight tissue equivalent
- Spiked in 5 fmol or 500 amol each heavy-labeled peptide standard
- Triplicate analyses

Antibody capture of heavy and light peptides
- PTMScan® Direct Multipathway reagent

Targeted LC-MS analysis
- Time-segmented SIM scan method on Q Exactive™ MS
- Monitoring 52 heavy/light pairs

Measured L/H ratio to determine response to each peptide

52-plex immunoassay provides successful quantification of key signaling protein phosphorylation from biopsy-level amounts of xenograft tumor tissue

Figure 8. Application of LC-MS immunoassay to H3255 xenograft tumor tissue. Left panel: Experimental details and flowchart. Right panel: Representative data are shown, with observed target peptide amount plotted against input xenograft wet weight tissue amount. Linear curve fits for each peptide are presented. Error bars represent standard deviation of triplicate immunoassays. A value of 0.1 fmol observed target peptide at the 400 mg input tissue level would be equivalent to 300 copies per cell if xenograft weight is assumed to consist entirely of H3255 cells.

Conclusions

- LCMS-SIM method is linear across five orders of magnitude
- Immunoassay combining PTMScan Direct phosphopeptide enrichment with LCMS-SIM method is robust and sensitive
- LCMS immunoassay applied to cell line and xenograft tumor tissue
- Assay sensitivity is sufficient for core tumor biopsy biomarker development

Contact Information

Kimberly A. Lee, Cell Signaling Technology, Inc. **Email:** klee@cellsignal.com **web:** www.cellsignal.com

Cell Signaling TECHNOLOGY®