MARQUIS: A Multiplex Method for Absolute Quantification of Peptides and Post-Translational Modifications

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Abstract

Absolute quantification of protein expression and post-translational modifications by mass spectrometry has been challenging due to a variety of factors, including the potentially large dynamic range of phosphorylation response. To address these issues, we have developed MARQUIS — Multiplex Absolute Regressed Quantification with Internal Standards — a novel mass spectrometry-based approach using a combination of isobaric tags and heavy-labeled standard peptides to construct internal standard curves for peptides derived from key nodes in signal transduction networks. We applied MARQUIS to quantify phosphorylation dynamics within the EGFR network at multiple time points following stimulation with several ligands, enabling a quantitative comparison of EGFR phosphorylation sites and demonstrating that receptor phosphorylation is qualitatively similar but quantitatively distinct for each EGFR ligand tested. MARQUIS was also applied to quantify the effect of EGFR kinase inhibition on glioblastoma patient derived xenografts. MARQUIS is a versatile method, broadly applicable and extendable to multiple mass spectrometric platforms.

Introduction

Innovations over the past decade in methodology and instrumentation have greatly increased the capability of mass spectrometry to identify a significant fraction of the proteome, including thousands of post-translational modifications (PTMs)\textsuperscript{1}. Through the use of quantitative strategies, including label-free proteomics, metabolic stable isotope labeling,
and chemical stable isotope labeling, it is possible to analyze two or more biological samples and generate relative quantification data detailing differences in protein expression or modification across different cellular conditions\(^2\,^3\). Multiplex labeling has effectively improved the throughput of the approach, enabling the comparison of multiple different biological samples in a single analysis, with quantification typically determined relative to a basal condition or pooled standard. Multiplexed relative quantification has been useful for elucidating temporal dynamics of phosphorylation signaling following growth factor stimulation, with changes in protein PTM levels in stimulated vs. non-stimulated conditions highlighting pathways involved in signal processing and cellular response\(^4\). When combined with bioinformatic algorithms, such as clustering, relative quantification of PTM level has enabled researchers to predict the function of poorly characterized phosphorylation sites\(^5\). Through quantification of downstream biological response and statistical analysis, key sites have been identified as regulators of observed cellular behavior, providing further functional and phenotypic association for novel phosphorylation sites\(^6\).

Despite the ability to generate biological insight with a combination of relative quantification of phosphorylation dynamics and statistical modeling or bioinformatics, additional information is encoded in the absolute levels of site-specific phosphorylation. For instance, relative fold change of a signal carries limited information about the network’s basal state or response to stimuli, as a two-fold change from a high basal state (e.g. 30% to 60% activation) can lead to a very different biological response from a two-fold change from a very low basal state (e.g. 1% to 2% activation). Absolute quantitation also enables comparison between multiple phosphorylation sites on a given protein under different conditions. Since each phosphorylation site might recruit adaptor proteins associated with particular signaling pathways, absolute quantification of phosphorylation can provide critical data relating receptor stimulation and pathway activation.

Absolute quantification of phosphorylation incorporation has historically been performed by using radiolabeled ATP and tryptic digestion followed by 2D thin-layer chromatography; however it is difficult to identify the specific phosphorylation sites corresponding to each peptide, the method is limited to single protein analysis, and the phosphorylation reaction must occur in vitro (in cell lysates) or in cells whose membranes have been disrupted. In considering more high-throughput techniques, absolute quantification data is not typically available from standard mass spectrometry, western-blot, or reverse-phase protein array experiments, as the calibration curve is unique to each phosphorylated peptide due to different response profiles (e.g. ionization potential or antibody binding affinity). Therefore multiple methods for estimating absolute quantification have been established. Among these, one of the most common MS-based methods is isotope dilution, or AQUA, in which a synthetic isotope-encoded peptide is added to the sample pre-analysis, and quantification is based on the relative peak heights or area under the chromatographic elution curve for the endogenous and synthetic peptide standard\(^7\). Although this technique is fairly straightforward, it relies on single point calibration and may provide erroneous estimates if the endogenous peptides span a large dynamic range across multiple conditions, or if the concentrations of these peptides fall outside of the linear response range of the instrument. Alternate methods for absolute quantification include the generation of standard curves through separate analyses of standard peptides at known concentration\(^7\). Since the complex
background of the biological context can significantly alter the signal intensity of the endogenous peptide due to competition for charge in the ionization process in MS, comparison to a standard curve generated in a neat background can lead to significant estimation errors. One solution would include different concentrations of a standard peptide into the sample so that the standard and endogenous peptides experience the same local sample context, but then one is faced with the issue of differentiating the standards from the endogenous peptides. Ideally, one would like to combine the multiplexed capabilities of chemical labeling with standard curves internal to the sample, thus allowing the accurate, absolute quantification of given peptides across multiple biological conditions within a single analysis.

With these goals in mind, we have developed Multiplex Absolute Regressed Quantification of Internal Standards (MARQUIS), an MS-based technique to measure the absolute amount of modification on many post-translational sites of interest simultaneously from multiple samples within a single analysis. The method utilizes multiple doses of internal standards to bracket a broad abundance range allowing simultaneous analysis of treatment conditions expected to produce widely varied endogenous signal magnitudes. We applied MARQUIS to quantify the absolute amount of phosphorylation on several sites in the Epidermal growth factor receptor (EGFR) network at different time points following stimulation of EGFR with different ligands. Absolute quantification of phosphorylation dynamics in this system highlights novel wiring within the network, insight that was not previously available from relative quantification data. MARQUIS enables the acquisition of accurate absolute quantification data, is applicable across multiple instrument platforms, and is equally applicable to protein expression profiling and PTM quantification.

Results

Assessing the requirement for standard peptides

To assess the need for internal standards, we selected several tyrosine phosphorylation sites in the EGFR signaling network, generated synthetic phosphorylated peptides encompassing these sites, and added these peptides at defined concentrations to cell lysate of MCF10A cells. Quantification of the signal intensity for each peptide across multiple different analyses demonstrated a significant amount of run-to-run variation (three representative examples are presented in Figure 1), likely associated with small flow rate fluctuations combined with differences in temporal sampling across the chromatographic elution profile. To quantify this data across multiple concentrations of each peptide, we calculated the response ratio, the ratio between signal amplitude and peptide concentration, for 13 peptides in the EGFR signaling network (Supplementary Fig. 1). As the data demonstrates, for a given concentration, the response ratio can vary by two to three orders of magnitude. This variation is likely associated with differences in ionization potential of the peptides and their respective co-eluting species, due to competition for charge in electrospray ionization. Together, the broad range in peptide-specific response ratio and the technical replicate variation make it difficult to directly relate signal intensity and abundance, thereby highlighting the need for an improved method to generate accurate absolute quantification data.
The MARQUIS method for absolute quantification

To quantify the absolute levels of endogenous peptides across a broad dynamic range, we have developed the MARQUIS (Multiplex Absolute Regressed Quantification of Internal Standards) method. Absolute quantification in MARQUIS is based on comparison of the endogenous peptides to paired heavy-isotope labeled internal standards added at specific concentrations into multiple biological samples (Fig. 2). Briefly, synthetic peptides containing a heavy-isotope labeled amino acid were generated for each phosphorylated peptide of interest and stock concentrations were established by amino-acid analysis (AAA). A standard peptide cocktail was then added to cell lysates; one concentration per lysate. These lysates were purified and digested prior to stable isotope labeling (iTRAQ) and combination. The mix of endogenous and synthetic peptides was subjected to targeted tandem mass spectrometry for iTRAQ quantification and peptide sequence confirmation. Sequence specific transitions were chosen based on previously observed fragmentation of the same peptides. Isobaric phospho-isoforms of the same peptide did not co-elute, enabling quantification of each isoform. Fragmentation of the iTRAQ-labeled synthetic, heavy-labeled phosphopeptide produced an internal standard curve with each calibration point corresponding to the unique amount of synthetic peptide added to the corresponding biological sample. Similarly, fragmentation of the iTRAQ-labeled endogenous peptide also produced iTRAQ marker ions, with the intensity of each ion corresponding to the amount of endogenous peptide in each biological sample. Calibration of the iTRAQ marker ion intensities from the endogenous peptide against the standard curve provided absolute quantification for each biological condition. The internal standard curve establishes the linear dynamic range around the endogenous peptide concentration, providing an accurate intrinsic quality assessment of the peptide quantification.

Since synthetic peptides are added early in the processing workflow, losses are identical for the endogenous and synthetic peptides as both experience identical environments through all post-digestion stages of preparation. Moreover, since the endogenous and heavy-labeled peptides co-elute, the peptides experience identical chromatographic and ionization contexts, removing an additional potential source of quantification error.

Performance of the method

To assess the performance of the MARQUIS method, heavy-labeled synthetic phosphorylated peptides were generated for selected tyrosine phosphorylation sites in the EGFR signaling network. Synthetic peptides were added to EGF-stimulated MCF10A cell lysate, thereby providing a complex, biologically-relevant background for testing the response profile, linear dynamic range, and sensitivity of this method. MARQUIS was initially evaluated on a triple quadrupole instrument operating in multiple reaction monitoring (MRM) mode, with 10 precursor-fragment ion transitions per peptide, including 2 sequence specific and 8 iTRAQ marker ion transitions (Supplementary Figs. 2, 3 and Supplementary Table 1). It is important to note that the number of phosphorylation sites able to be monitored with this approach is inherently limited by the number of transitions. With 10 transitions per peptide and 2 peptides (synthetic and endogenous) per phosphorylation site, the total number of transitions increases rapidly as more sites in the network are monitored. Increasing the number of transitions translates to fewer measurements per...
peptide across the chromatographic elution profile, leading to sub-optimal quantitative data. To address this issue, we also evaluated MARQUIS on an Orbitrap-based instrument (QExactive) using targeted tandem mass spectrometry, also known as parallel reaction monitoring (PRM). In this approach, a full scan, high-resolution MS spectrum is acquired followed by full scan, high-resolution MS/MS spectra containing both iTRAQ marker ions and sequence specific peptide fragmentation for each peptide (Supplementary Fig. 4). Since the endogenous and heavy-labeled peptides are both detected in the MS scan, quantitative comparison of the precursor ion intensities provides an estimate of the total amount of endogenous peptide present in the sample (Supplementary Table 2). The relative iTRAQ marker ion intensities from the MS/MS spectrum for the endogenous peptide then apportion this total amount across the biological samples. iTRAQ marker ion intensities from the MS/MS of the standard peptide serve as an important control by providing a peptide-specific estimate of the accuracy and linear dynamic range. For both the MRM and PRM methods, we selected 13 phosphorylation sites to assess the performance of MARQUIS quantification at different peptide concentrations (full data available in Supplementary Table 3, representative temporal data for two peptides are plotted in Supplementary Fig. 5).

Representative data extracted for two standard peptides using MRM and PRM is shown in Figure 3. At the high end of the curve, both methods perform identically. For EGFR pY1173 (Fig. 3a), the linear range for both MRM and PRM extends from 3000 fmol to 100 fmol; between 100 fmol and 3 fmol the response remains linear, but deviates from the theoretical response due to additional noise in the experimental measurement. For Shc pY317 (Fig. 3b) the PRM method performs well with a dynamic range of approximately 1000-fold (3000 fmol to 3 fmol). The MRM method for this site did not perform as well, with suspected contamination on the iTRAQ-118 (10 fmol) channel boosting the recorded intensity significantly. This contamination does not appear to affect the iTRAQ-119 (3 fmol) or iTRAQ-121 (1 fmol) channels as both show measurably lower values than iTRAQ-117 (30 fmol). Contamination may appear in the MRM method and not the PRM method due to the ability of the high resolution PRM method to distinguish contaminant ions from iTRAQ marker ions, whereas the MRM measurement detects the number of ions passing a set bandwidth in the third quadrupole. It is worth noting that altering the dwell time per transition for MRM or the ion target value and maximum fill time for the PRM method can affect the sensitivity of the analysis and thereby improve the linear dynamic range, but increasing these values comes at the expense of cycle time.

To evaluate the reproducibility of quantification using the MARQUIS method, we performed technical replicates of each experiment on the MRM and PRM platforms and plotted the concentration vs. coefficient of variation (CV) for each measurement in the data set (Fig. 3c). Although both methods provide high quality data (e.g. most measurements have CV <0.15) across the entire range, the most reproducible measurements are obtained above 100 fmol, with CV values <0.1. At the low end of the concentration range, some peptides display CV >0.5. For MRM, the early time points of ERK1 and ERK2 signals exhibited the highest CV, likely due to the combination of low endogenous signal coupled to the higher standard doses included in the corresponding channels. Inclusion of a second heavy-labeled amino acid residue would increase the spacing between the endogenous and standard peptides and thereby decrease the potential issue of isolation window.
contamination. The highest CV’s for the PRM technique were those of the dual phosphorylated EGFR peptide pY1045/pY1068. This peptide has a lower response ratio (Supplementary Fig. 1) and elutes very late in the chromatographic program, making it more susceptible to contamination from background chemical noise. Overall, both techniques were comparable in terms of the fraction of measurements that met CV thresholds (Fig. 3d) at a CV of 0.20.

**Isolation Width Effects**

In the MARQUIS method, both the endogenous and the heavy-labeled synthetic peptides are iTRAQ labeled, allowing for direct comparison of iTRAQ marker ion intensities resulting from fragmentation of the corresponding peptides. This approach has the potential for error associated with contamination of the isolation window from other endogenous or synthetic peptides, or potentially from co-isolation of the endogenous and heavy-labeled peptides. As the charge state of the peptide increases, the separation, in m/z, between the endogenous and heavy-labeled synthetic peptide decreases, thereby increasing the likelihood of co-isolation.

To assess the effect of the isolation width on MARQUIS quantification accuracy for the MRM platform, we narrowed the isolation width on the first quadrupole from 0.7 m/z to 0.1 m/z. Narrowing the MS1 isolation window can decrease the level of contamination in the ions selected for fragmentation, but also significantly decreases the transmission efficiency. To determine if this trade-off was beneficial, we compared the measured signal intensities from all peptides from analyses run with both isolation widths (Supplementary Fig. 6). Minimal effects of altered isolation width were seen for the highest peptide levels, whereas only the 0.1 m/z isolation window was able to clearly distinguish the median of the 1 fmol standard from the median of the 10 fmol standard. At the lowest peptide levels, the narrower isolation width was better able to isolate precursor ions, ultimately providing a larger dynamic range for calibration. However, for peptides with poor response ratios, the losses associated with the narrower isolation width ultimately limit the sensitivity and therefore the dynamic range.

**Tyrosine Phosphorylation Quantification in EGFR signaling**

Previous studies have described the temporal dynamics of individual tyrosine phosphorylation sites following EGFR activation\(^8\). Although these studies have highlighted many novel phosphorylation sites in the network and have led to hypotheses regarding the function of selected sites, they have been inherently limited by quantification relative to a selected time point. As such, it has been difficult to quantify differences in phosphorylation levels across different sites on the receptor, or to determine whether different ligands led to different phosphorylation stoichiometries. To address this deficiency and gain insight into the temporal phosphorylation profiles of multiple sites in the network, we used MARQUIS to quantify absolute levels of tyrosine phosphorylation on EGFR and proximal adaptor proteins, at multiple time points following EGF stimulation. Although EGFR pY1173 is often cited as a dominant autophosphorylation site for the receptor\(^9\), and is often used as a measure of EGFR activation for immunoblotting studies\(^10,11\), MARQUIS data unequivocally demonstrates that phosphorylation of 1148 occurs approximately 4–5 fold greater than pY1173, pY1068, or pY1045, all of which occur at similar levels (Fig. 4a), a comparison that would be missed by typical MS-based relative quantitation\(^6\) or
immunoblotting techniques. Further downstream in the network, it is also possible to detect higher levels of phosphorylation on Erk2 pY187 as compared to Erk1 pY204 (Fig. 4b), an observation that aligns with higher ERK2 expression in other systems.  

Absolute quantification enables the tracking of individual phosphorylation sites, even when multiple sites are contained within the same tryptic peptide. For instance, the peptide containing Y1148 also contains S1142, a serine residue that has previously been found to be phosphorylated in these cells. To identify whether pS1142 co-occurs with pY1148 on the same EGFR protein, we used MARQUIS for absolute quantification of the singly phosphorylated pY1148 peptide and the doubly phosphorylated pS1142/pY1148 peptide. By quantifying the singly and doubly phosphorylated isoforms of the peptide, we could more accurately account for the total amount of phosphorylation on Y1148 (Fig. 4c). This analysis led to the intriguing finding that pS1142/pY1148 is present in unstimulated cells at approximately the same level as the singly phosphorylated pY1148, but upon stimulation gradually decreases over time. In comparison, the singly phosphorylated pY1148 increases strongly following EGF stimulation. With absolute quantification, it is clear that the increase in phosphorylation of the singly phosphorylated pY1148 is much greater than the decrease in the doubly phosphorylated pS1142/pY1148 peptide. Therefore, the increase in singly phosphorylated pY1148 is likely due to phosphorylation of EGFR in the absence of pS1142, rather than de-phosphorylation of the pS1142/pY1148 species. It remains to be determined whether pS1142 inhibits phosphorylation of Y1148, but our data would be consistent with this hypothesis.  

Additional insights into phosphorylation dynamics are available when multiple tyrosine phosphorylation events occur on a given tryptic peptide. For instance, Y1045 and Y1068 fall within the same tryptic fragment, and therefore yield four possible phosphorylation isoforms for the peptide (Y1045/Y1068, pY1045/Y1068, Y1045/pY1068, and pY1045/pY1068), three of which were monitored by MARQUIS-based absolute quantification (Fig. 4d). Although all three isoforms are present at similar, low levels in unstimulated MCF10A cells, following stimulation Y1045/pY1068 and doubly phosphorylated pY1045/pY1068 both rise rapidly in the first minute. The pY1045/Y1068 peptide increases gradually in the first minute, potentially indicating that this site is being converted to the pY1045/pY1068 form through rapid phosphorylation of Y1068. Interestingly, over the next several time points the singly phosphorylated Y1045/pY1068 isoform begins to decrease, while the pY1045/Y1068 and pY1045/pY1068 isoforms both increase strongly, indicating phosphorylation of Y1045 that is converting Y1045/pY1068 into the doubly phosphorylated form, which is the most abundant of the three isoforms in this early time period. Finally, by five minutes all three of the phospho-isoforms begin to decrease at similar rates, potentially indicating the activity of a common phosphatase. The quantitative interplay between these isoforms has not been previously documented due to a lack of absolute quantification of phosphorylation level on each of these sites under different stimulation conditions.  

MARQUIS-based absolute quantification was also used to quantify EGFR phosphorylation dynamics in response to different EGFR ligands. It has previously been documented that stimulation with Epidermal Growth Factor (EGF), Transforming Growth Factor α (TGFα), or Amphigirinulin (AREG) leads to different downstream biological effects, potentially
due to qualitatively or quantitatively different signaling networks. To uncover the mechanism by which these different ligands activate different phenotypes, we quantified the temporal dynamics of phosphorylation levels on multiple sites on the receptor and several downstream adaptor or effector proteins in response to different concentrations of each ligand (Supplemental Table 4). As can be seen in Figures 5a–c, each ligand stimulates a rapid increase in receptor phosphorylation, albeit to different levels, with the Y1148 site consistently phosphorylated to a greater extent compared to the other three phosphorylation sites on the C-terminal tail of the receptor. Given that each ligand has different binding affinities, the difference in the level of phosphorylation on the receptor may not be surprising. Intriguingly, despite the quantitative difference in the level of phosphorylation due to each ligand (Fig. 5d), the pattern of phosphorylation on the receptor is effectively invariant with regard to ligand identity, with pY1148>pY1068>pY1173>pY1045; this similarity in receptor pattern can best be visualized by normalizing all sites for a given ligand to the total amount of phosphorylation on the receptor (Fig. 5e). The consistent pattern of receptor phosphorylation at this ligand dose raises the intriguing possibility that biological response to different EGFR ligands may be encoded in the absolute level of phosphorylation on each site, rather than through the phosphorylation of different sites on the receptor (e.g. quantitative vs. qualitative control of RTK phosphorylation signaling networks). Interestingly, quantitative control at the receptor level results in altered phosphorylation patterns even on proximal adaptor proteins such as Gab1 (Supplementary Table 4); the mechanism underlying these pattern changes is currently unknown, but it is likely that changes in the amount of phosphorylation at the receptor may affect recruitment of other enzymes (kinases/phosphatases) or stearic competition for binding to the receptor, thus leading to different protein complex assembly.

Quantification of EGFR signaling in Glioblastoma Xenografts—To assess the applicability of the MARQUIS method to the complex biological background represented by tissue samples, we chose a set of patient-derived xenografts (PDX) that vary in their EGFR expression and mutation profiles: GBM12 expresses a point mutation the extracellular domain of EGFR; GBM6 and GBM39 express the constitutively active truncation mutant EGFR vIII; and GBM15 is EGFR wild-type amplified. Before searching for the endogenous signals, we confirmed that we could still build a large-dynamic-range standard curve in the more complex tumor background (Supplementary Figure 7 and 8). In the worst cases, we were still able to cover at least two orders of magnitude, convincing us that we could adequately measure endogenous levels.

With MARQUIS in PRM mode, we compared the absolute levels of phosphorylation for each EGFR phosphorylation site both within each PDX tumor model and across the various tumor models (Figure 6a and Supplementary Table 6, (validated spectra for each quantification scan are included in Supplementary Figure 9a–d)). For these PDX tumors, GBM6 (EGFRvIII) had the lowest level of EGFR phosphorylation, consistent with previous literature describing EGFRvIII as a weakly constitutively active kinase. Interestingly, GBM39 also expresses EGFRvIII, but is much more highly phosphorylated on the receptor. By comparison, GBM12 (EGFR point mutant) demonstrated an intermediate level of receptor phosphorylation and GBM15, the wild-type EGFR amplified PDX tumor, had the
highest level of phosphorylation. Large variation was observed in the different GBM15 tumor samples, potentially due to biological fluctuations between replicate tumors. Intriguingly, the pattern of EGFR phosphorylation was invariant when comparing the wild-type EGFR (GBM15) to the point mutant (GBM12) or the EGFRvIII mutation (GBM6 and GBM39), providing a separate example of quantitative vs. qualitative regulation of receptor phosphorylation. For proximal adaptor proteins, little significant difference was observed in Gab1 Y406 phosphorylation (Figure 6b), but Shc pY317 levels (Fig. 6c) were elevated for GBM12 and GBM 39 relative to GBM15 and GBM6. Downstream of SHC and GAB1, ERK2 was most highly phosphorylated in GBM12 and GBM15, while ERK1 phosphorylation levels were similar across all four GBM PDX tumors (Figure 6d). It is worth noting that this data is in general agreement with our previous analysis of the signaling networks in these GBM PDX tumor lines, except that MARQUIS provides absolute levels of quantification for the specific phosphorylation sites in each sample.

As an additional application, MARQUIS enables the absolute quantification of the effects of therapeutic treatment on tumor samples. In many cases, it is not known whether resistance arises due to failure to inhibit the target or due to compensatory signaling mechanisms. To quantify the effect of EGFR tyrosine kinase inhibitor treatment in vivo, GBM12 was treated with a panel of kinase inhibitors (Erlotinib, Dacomitinib, or NT-113), to compare the efficacy of a classic Type 1 inhibitor (Erlotinib) with the latest generation of covalent HER-family inhibitors (Dacomitinib and NT-113). Following tumor homogenization, the MARQUIS workflow was employed in PRM mode with the same heavy-labeled standard peptides and full-scan data collection LC-MS (validated spectra for each quantification scan are included in Supplementary Figure 8). The results of these analyses are presented in Figure 7 and data is in Supplementary Table 7. EGFR phosphorylation (Fig. 7a) in the placebo treated tumors continues to resemble the patterns observed in the MCF10A cells treated with EGF. Following treatment with each of the inhibitors, EGFR phosphorylation on each site decreased significantly, by >90% on pY1068, pY1148, and pY1173, indicating effective inhibition of the target for each kinase inhibitor. Phosphorylation on the proximal adaptor proteins Gab1 pY285, Gab1 pY659 (Fig 7b), and Shc pY317 (Fig. 7c) also decreased in response to each of the inhibitors, which corresponded to a decrease in Erk1 and Erk2 phosphorylation (Figure 7d). None of the inhibitors had a significant impact on Src Y418 phosphorylation, but NT-113 decreased Shp2 phosphorylation on Y62 to a greater extent (~44% decrease) compared to dacominitib (~22%) and erlotinib (~10%).

**Discussion**

MARQUIS is a novel multi-point internal standard calibration method that enables absolute quantification of site-specific PTM levels or protein expression. The method provides accurate quantification over a broad range of concentrations allowing simultaneous analysis of stimulation conditions that lead to large changes in expression or modification levels. With MARQUIS, absolute quantitation data can be collected for many PTM sites of interest in the network in a single analysis, using either MRM or PRM-based targeted mass spectrometry. This data can then be used for intra- and inter-sample site-specific comparisons.
It is well known that isobaric labeling strategies such as iTRAQ or TMT suffer from ratio compression in complex mixture analysis due to the inclusion of contaminating peptides during the isolation of the precursor peptide for fragmentation. Importantly, the MARQUIS method is novel in the inclusion of a standard curve within the biological sample, enabling the estimation of ratio compression at the same elution time as the endogenous peptides, and at a proximal mass-to-charge ratio. Thus, the MARQUIS method not only correctly estimates iTRAQ ratio compression, but also enables compensation for that compression, a feature that is inherently lacking in most other absolute or relative quantification methods.

It is also worth noting that the MARQUIS method enables accurate absolute quantification even for complex sample processing protocols prior to mass spectrometric analysis. Because synthetic phosphorylated peptides whose sequence matches exactly to the endogenous peptide are added during the cell lysis procedure, the MARQUIS method accounts for losses throughout post-digestion sample processing, including potentially inefficient immunoprecipitation of selected peptides or sub-optimal enrichment of phosphorylated peptides.

MARQUIS was designed for MRM-based analysis on a triple quadrupole, but the method is equally applicable to targeted full-scan targeted tandem mass spectrometry (PRM). Overall, the two methods performed very well in terms of technical reproducibility with a CV threshold of 0.15 able to capture the majority of measurements with both techniques. Both instrument platforms offer selective advantages. For instance, the PRM method offers the ability to determine contamination by way of the complete MS/MS data at high resolution that is not available in an MRM experiment. On the other hand, when using MRM, the precursor (Q1) isolation window can be tightened to a greater extent compared to the PRM method, thereby improving signal-to-noise by reducing contributions from spurious co-eluting ions. Although a Q1 isolation width of 0.7 m/z was typically used for the data acquired in this study, sufficient signal for quantification can be attained for many peptides by using a window as small as 0.1 m/z. Such a narrow window would be deleterious to the PRM method as the drop in ion flux may eliminate signal for most low and medium intensity fragments. The MRM approach is inherently more flexible, as the fragmentation energy and time spent per MRM transition can be altered for selected transitions to improve the sensitivity. However, increasing the dwell time is only feasible for a small number of transitions; otherwise the increase in cycle time becomes too great.

Recent structural insights into the cooperativity of ligand binding and EGFR dimerization suggest that differential ligand affinities for the extracellular portion of the receptor influence the occupancy and the receptor conformation and potentially signaling and trafficking. A previous mass spectrometry-based study of receptor phosphorylation observed site-specific quantitative differences in receptor phosphorylation in response to varied doses of TGFα and EGF; stoichiometry was estimated by measuring the ratio of signal intensities between phosphorylated and non-phosphorylated versions of tyrosine containing tryptic fragments. Such comparisons are limited due to the differential ionization potential between these species. Here we have shown large differences not only between various ligand stimulation conditions for a single site, but also between different EGFR sites not present on the same tryptic peptide.
The MARQUIS method enables quantification of EGFR phosphorylation stoichiometry, information necessary to decipher how extracellular ligand concentration information is transmitted across the cellular membrane. Unique sets of SH2- and PTB-containing proteins bind to each receptor phosphorylation site, suggesting the ability to activate distinct pathways by phosphorylating different sites. Alternately, differential pathway activation could be controlled by altering the level of phosphorylation. While this option might suggest that downstream pathways will be linearly proportional to receptor phosphorylation levels, different pathways feature amplification gains, and thus one pathway might be saturated by a relatively small signal on the receptor, as has been detected for the ERK MAPK pathway, while other pathways may be more analog in nature. Here, through absolute quantification of specific phosphorylation sites on the receptor, we determined that the EGFR phosphorylation response to stimulation with a relatively high concentration of different EGFR ligands is quantitatively regulated. Although phosphorylation levels are ligand specific, the ratio between different receptor sites is constant across different EGFR ligands. Thus, different biological responses may be regulated by the analog vs. digital nature of the different downstream pathways, in a manner analogous to the interleukin receptor system we have recently investigated. Additional studies are needed to confirm the effects of quantitative regulation of receptor phosphorylation on downstream pathways, as well as to determine the cellular conditions that might lead to altered phosphorylation patterns on the receptor: is this a mechanism by which the cell differentiates normal vs. pathological signaling?

Finally, we have demonstrated the applicability of the MARQUIS method to generate absolute phosphorylation quantification data in tumor tissues. Because MARQUIS provides absolute quantification in a multiplexed analysis, it enables quantification across different MS analyses, now providing the potential to generate absolute quantification of hundreds of tumor samples in a relatively small number of MS analyses. Furthermore, we have used MARQUIS to quantify the effects of therapeutic inhibition on these same tumor tissues. These data demonstrate the ability of MARQUIS to not only quantify the effect of different TKIs on target phosphorylation levels, but also to quantify the effect of the inhibitor on proximal adaptor proteins, downstream effectors, and off-target kinases.

Absolute quantification of protein expression and PTM levels will be required to provide insight into regulation of many biological systems. The MARQUIS method enables facile acquisition of absolute quantification data across multiple biological conditions simultaneously, with large dynamic range, and with internal standard curves to improve the accuracy of the measurements. This method is broadly applicable to multiple mass spectrometry platforms and to different biological systems.

**Methods**

**Sample Preparation**

MCF10A cells (originally provided by the Brugge lab at Harvard Medical School) were serum-starved 24 hours prior to stimulation with 20nM EGF (Peprotech) for 0.5, 1, 2, 3, 5, 10 or 30 minutes. Media was aspirated and cells were lysed with 2ml of cold 8M urea with 1mM activated sodium orthovanadate. Lysate was frozen at −80 until further processing.
Protein yield was quantified by BCA assay (Pierce) in order to ensure equal loading. Standard peptides were added to samples as in Supplementary Table 1. Samples were reduced with 40µl 10mM DTT in ammonium acetate pH 8.9 for 1 hour at 56 C. Samples were alkylated with 55mM iodoacetamide in ammonium acetate pH 8.9 for 1 hour at room temperature. 8mL ammonium acetate and 40µg of sequencing grade trypsin was added prior to 16 hours incubation at room temperature. Lysates were acidified with 1 mL glacial acetic acid and protein was purified with Waters Sep-Pak columns. Samples were lyophilized and subsequently labeled with iTRAQ 8plex (AbSciex) per manufacturer’s directions.

Standard Peptide Preparation and Quality Assurance

Standard peptides were synthesized at the Koch Institute Biopolymers facility and amino acid analysis was performed to measure concentration by AAA Service Laboratories (Damascus, OR). Synthetic peptides were analyzed by LC MS/MS to confirm sequence and purity.

Multiple Reaction Monitoring (MRM)

**Immunoprecipitation**—70ul protein G agarose beads (calbiochem IP08) were rinsed in 400ul IP Buffer (100mM tris, 0.3% NP-40, pH 7.4) prior to an 8 hour incubation with a cocktail of three phosphotyrosine-specific antibodies (12µg 4G10 (Millipore),12ug PT66 (Sigma), and 12ugPY100 (CST)) in 200ml IP Buffer. Antibody cocktail was removed and beads were rinsed with 400ul of IP Buffer. Labeled samples were resuspended in 150ul iTRAQ IP Buffer (100mM Tris, 1% NP-40, pH 7.4) + 300ul milliQ water and pH was adjusted to 7.4 (with 0.5M Tris HCl pH 8.5) prior to addition to prepared beads and 16 hour incubation. Supernatant was removed and beads were rinsed 3 times with 400ul Rinse Buffer (100mM Tris HCl, pH 7.4). Peptides were eluted in 70ul of Elution Buffer (100mM glycine, pH 2) for 30 minutes at room temperature.

**Immobilized Metal Affinity Chromatography (IMAC) Purification**—A fused silica capillary (FSC) column (200µm ID×10cm length) was packed with POROS 20MC beads (Applied Biosystems cat.no. 1-5429-06). Column was rinsed with 100 mM EDTA pH 8.9 in ultrapure water to remove residual iron and then rinsed with ultrapure water to remove EDTA before fresh iron was deposited by rinsing with 100 mM FeCl$_3$ in ultrapure water. Excess iron was removed with 0.1% acetic acid prior to loading the IP elution. The column was washed with 25% MeCN, 1% HOAc, 100 mM NaCl in ultrapure water to remove non-specifically bound peptides and then rinsed with 0.1% HOAc. Peptides were eluted with 250mM NaH$_2$PO$_4$ in ultrapure water and collected on a pre-column (100µm ID × 10cm packed with 10µm C18 beads (YMC gel, ODS-A, 12nm, S-10 µm, AA12S11)). This pre-column was rinsed with 0.2M acetic acid prior to LCMS analysis.

**Liquid Chromatography Mass Spectrometry**—The pre-column was connected in-line with an analytical column (50µm ID × 10cm packed with 5µm beads (YMC gel, ODSAQ, 12nm, S-5 µm, AQ12S05)) with integrated electrospray emitter tip [Martin 2000]. A 140 minute gradient from aqueous (0.2M acetic acid) and organic phase (0.2M HOAc, 70% MeCN) was used to separate peptides with a flow rate of 200 nL/min and a flow split > 99% and a spray voltage of 1800 V. Isolation widths of 0.7 were used for both Q1 and Q3 and
collected for 0.05 seconds. A width of 0.01 m/z was collected around each transition for quantification. Collision energies were determined per sequence with Thermo Pinpoint software.

**Absolute Quantitation**—Analyses were performed on a Thermo TSQ Quantum in MRM mode. Endogenous and heavy-labeled versions of each peptide of interest were tracked throughout the experiment with two sequence specific transitions (precursor/prominent b or y ion) and eight iTRAQ transitions (precursor/label peak). Elution time was determined based on co-elution of iTRAQ transitions with sequence specific transitions for both standard and endogenous peptides of the MARQIS pair. Area Under the Curve (AUC) was collected for each transition. Data were corrected by iTRAQ isotope purity and synthetic peptide loading control. A calibration curve was created for each peptide to relate the AUC of the iTRAQ transition to the amount of standard included. MATLAB function `lscov` was used with the intensity of the signal also serving to appropriately scale the variance. This calibration curve was then used to calculate the amount of peptide present in each of the endogenous conditions based on the corresponding iTRAQ transition AUC.

**Parallel Reaction Monitoring (PRM)**

**Immunoprecipitation**—20 µl protein G agarose beads (Calbiochem, IP04-1.5ML) were pre-incubated with 20µl of PT66 and 20µl of pY100, prior to adding sample (reconstituted in 70µl HEPES buffer adjusted with 5M NaOH). After overnight incubation, beads were rinsed and peptides were eluted with 20µL of 0.1% TFA, 10% MeCN, 250ng/µL HeLa Digest (phospho depleted).

**NTA Enrichment**—Elution incubated with Fe(III)-NTA magnetic beads for 15 minutes, rinsed and eluted with 10µL of NH₄OH (1:30), 5mM EDTA, 5% MeCN, 1ng/µl HeLa digest. Elution was transferred to an autosampler vial and pH was adjusted with 5µl of 10% TFA.

**Liquid Chromatography Mass Spectrometry**—LC-MS was performed using a 25cm, 75µm ID, EASY-Spray column (ThermoFisher Scientific, ES802) with a 120 minute gradient and a flow rate of 300µl/min and a spray voltage of 1800 V. Targeted MS2 scans were collected with a maximum ion injection time of 2 seconds and an AGC target of 3e6 with an MS1 isolation width of 1 m/z and fragmented with a collision energy of 35.0.

**Absolute Quantitation (PRM method)**—Analyses were performed on a Thermo Q-Exactive. Endogenous and heavy-labeled versions of each peptide were targeted for MS2 analysis during their expected elution times. In the MS1 scan, the total amount of endogenous peptide was calculated based on the fraction of MS1 integrated intensity when compared to the standard peptide. This amount was then apportioned across the contributing samples based on relative iTRAQ intensity. A single point measurement where the MS2 scan used to determine the iTRAQ intensity ratios was chosen based on maximal MS1 intensity to assure maximal signal to noise ratio.
Tumor Sample Analysis

All studies were reviewed and approved by the Mayo Institutional Animal Care and Use Committee. Subcutaneous xenografts for GBM 6, 12, 15, and 39 were established by injecting the flank of athymic nude mice with $1 \times 10^6$ cells suspended in Matrigel/PBS. Tumors were harvested and flash frozen in liquid nitrogen once they reached $>1500$ mm$^3$ in size. For the GBM 12 pharmacodynamic study, eight mice had flank tumors implanted and randomized into four treatment arms once the tumor reached at least $200$ mm$^3$ in size: 1) Placebo 2) erlotinib $100$ mg/kg 3) dacomitinib $10$ mg/kg 4) NT-113 $15$ mg/kg. A single dose was given to each mouse and the tumor was harvested at six hours post-treatment and flash frozen in liquid nitrogen.

500 µg of protein (as determined by BCA assay) from each tumor sample was processed as per the PRM LC-MS protocol following IP and IMAC enrichment as previously described. Analyses were performed on a Thermo Q Exactive instrument fed by a Thermo Easy nLC1000 UPLC precolumn (Thermo 164705) and a self-pack PicoFrit analytical column (New Objective PF360-50-10-N-5) packed with 50 cm of 3µm beads (ODS-AQ 12nm S-3um from YMC America AQ12S03). Samples were analyzed on a 60 minute gradient with a 200 nL/min flow rate; aqueous phase: 0.1% Formic Acid in HPLC-grade water, organic phase: 0.1% Formic Acid, 80% Acetonitrile in HPLC-grade water. A spray voltage of 2000 V was used.

To ensure that the standard peptides did not contaminate the endogenous iTRAQ signals, each sample was run twice. Quantitative iTRAQ data for the endogenous peptides was collected from Sample A, as Sample A did not contain standard peptides that may potentially contaminate endogenous iTRAQ signal. MS/MS scans of endogenous peptides were collected with a 250 ms fill time, an AGC target value of 5e5 and an isolation window of 2 m/z. Quantitative iTRAQ data for the synthetic heavy-labeled standard peptides as well as the ratio between standard and endogenous precursor peaks were collected from Sample B. MS/MS scans were optimized to collected the largest dynamic range possible by maximizing the fill time (3s) and the AGC target (5e6).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Variability of Signal Intensity from Technical Replicates
The signal intensity vs. amount of peptide is plotted for three representative peptides across five technical replicate analyses.
Figure 2. MARQUIS Method for Absolute Quantification

Synthetic isotope-labeled phosphorylated peptides (represented by the square box) are added during cell lysis. Following sample processing and digestion, all peptides, synthetic and endogenous (represented by the circle), are iTRAQ labeled. For this particular application of the method, tyrosine phosphorylated peptides are enriched by peptide immunoprecipitation followed by immobilized metal affinity chromatography (IMAC) and analyzed by LC-MS/MS. Heavy-labeled standards provide an internal calibration curve, enabling absolute quantification of endogenous peptides.
Figure 3. Quality Comparison of MARQUIS on the MRM vs. PRM Platform

Observed vs. Expected total iTRAQ signal contribution for MRM and PRM methods for two sites: EGFR pY1173 (a) and Shc pY317 (b). c) Coefficient of Variation vs. Amount for MRM and PRM methods. d) Fraction of measurements that meet Coefficient of Variation (CV) thresholds for both techniques. Data represents mean and standard deviation across five technical replicates.
Figure 4. Stoichiometric Comparison of Site-Specific Phosphorylation
Data for a collection of EGFR and network phosphorylation sites was gathered using MARQIS with Multiple Reaction Monitoring (MRM) on a triple quadrupole instrument. a) EGFR. b) ERK1/2. c) EGFR pY1148, pS1142/pY1148. d) EGFR pY1045, pY1068, and pY1045/pY1068. Data represent mean and standard deviation across three biological replicates.
Figure 5. Ligand Comparison
MRM-based absolute quantitation for EGFR phosphorylation sites treated with EGF (a), TGFα (b), and AREG (c). Comparison of site-specific phosphorylation between ligands following five minutes of ligand stimulation for two biological replicates; raw (d) and normalized (e). Data represent mean and range across two biological replicates.
Figure 6. Quantification of Phosphorylation Across Four Glioblastoma Patient-Derived Xenograft Tumor Types
Absolute amounts of phosphorylation were quantified for each patient-derived xenograft mouse model of glioblastoma. Each tumor model was analyzed in biological duplicate. EGFR (a). Gab1 (b). Shc (c). Erk1/2 (d). Data represent mean and range across two biological replicates.
Figure 7. Quantification of Phosphorylation in a Glioblastoma Patient-Derived Xenograft Treated with a Panel of EGFR Kinase Inhibitors
Absolute amounts of phosphorylation were quantified in the GBM12 patient-derived xenograft tumors treated with placebo or one of three EGFR kinase inhibitors: Erlotinib (100 mg/kg), Dacomitinib (10 mg/kg), or NT-113 (15 mg/kg). Each treatment condition was analyzed in biological duplicate. EGFR (a). Gab1 (b). Shc (c). Erk1/2 (d). Data represent mean and range across two biological replicates.