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Assay Development for the Determination of Phosphorylation Stoichiometry using MRM methods with and without Phosphatase Treatment: Application to Breast Cancer Signaling Pathways

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Abstract

We have developed a phosphatase-based phosphopeptide quantitation (PPQ) method for determining phosphorylation stoichiometry in complex biological samples. This PPQ method is based on enzymatic dephosphorylation, combined with specific and accurate peptide identification and quantification by multiple reaction monitoring (MRM) detection with stable-isotope-labeled standard peptides. In contrast with the classical MRM methods for the quantitation of phosphorylation stoichiometry, the PPQ-MRM method needs only one non-phosphorylated SIS (stable isotope-coded standard) and two analyses (one for the untreated and one for the phosphatase-treated sample), from which the expression and modification levels can accurately be determined. From these analyses, the % phosphorylation can be determined.

In this manuscript, we compare the PPQ-MRM method with an MRM method without phosphatase, and demonstrate the application of these methods to the detection and quantitation of phosphorylation of the classic phosphorylated breast cancer biomarkers (ERα and HER2), and for phosphorylated RAF and ERK1, which also contain phosphorylation sites with important biological implications. Using synthetic peptides spiked into a complex protein digest, we were able to use our PPQ-MRM method to accurately determine the total phosphorylation stoichiometry on specific peptides, as well as the absolute amount of the peptide and phosphopeptide present. Analyses of samples containing ERα protein revealed that the PPQ-MRM is capable of determining phosphorylation stoichiometry in proteins from cell lines, and is in good agreement with determinations obtained using the direct MRM approach in terms of phosphorylation and total protein amount.

Keywords

MRM; PPQ; phosphorylation; quantitation; stoichiometry; signaling pathways

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Introduction

Reversible phosphorylation is one of the most common posttranslational protein modifications and is used to regulate protein activity by multicellular organisms.[1, 2] Current methods are able to predict and determine thousands of phosphorylation sites, but the stoichiometry and function of these sites cannot yet be predicted or accurately determined in "shotgun" proteomics experiments. Phosphorylation sites with 100% stoichiometry are likely to be extremely important.[3]; the importance of low levels of phosphorylation is still not understood. It has been said that sites with low phosphorylation stoichiometry may be "biochemical noise".[4] Conversely, it has been proposed that low stoichiometry may represent a less stable, faster mechanism of protein activation.[5] Determination of the stoichiometry of phosphorylation may be as significant as the location of the phosphorylation site, but it is not possible to determine the *importance* of phosphorylation stoichiometry without being to accurately *determine* the phosphorylation stoichiometry. This is the current challenge in phosphoproteomics, and why we have developed a new multiplexed high-throughput method for the accurate absolute quantitation of phosphorylation.

Although mass spectrometry is currently the method of choice for identifying phosphorylation sites, phosphopeptides, especially multiply-phosphorylated phosphopeptides, have lower sensitivities than their non-phosphorylated counterparts. This causes difficulties in both MS-based phosphorylation site mapping and quantitation. The facile loss of the phosphoryl group and/or the elements of phosphoric acid poses additional challenges to MS and MS/MS-based quantitation. Recent studies have even demonstrated that the sites of phosphorylation within a peptide shift during CID-MS/MS creating significant ambiguity in the assignment of correct phosphorylation sites in phosphopeptides containing multiple potential sites of phosphorylation.[6, 7]

The removal of the phosphoryl group by means of alkaline phosphatase has been successfully used to improve the detection of formerly-phosphorylated peptides in both MALDI [8-13] and electrospray ionization.[14] Determining relative phosphorylation stoichiometry without the use of standard phosphorylated peptides is usually done by comparing the peak heights or peak intensities of the phosphorylated and non-phosphorylated forms. To compare two experimental treatments, the non-phoshorylated form is used as an internal standard. While this has the advantage of being applicable to "shotgun" methods where the target proteins are not known in advance, it makes the assumption that the amount of the non-phosphorylated isoform is unchanged between the two treatments. A variant of this technique, where the assumption is made that the total amount of this potentially-phosphorylated protein remains the same, is to use non-phosphorylated peptides from the same protein as internal standards. Ruse et al. [15] used this method (which they termed "the native reference peptide method") with selected ion monitoring of the phosphorylated and non-phosphorylated $(M+H)^+$ ion. One approach to solving the problem of variability of the potentially phosphorylated protein between the treatments, is to either use a different (assumed to be non-changing) protein as an internal standard, or to spike an internal standard into the digests to be used as an internal standard [16]. The disadvantages of all of these techniques are that the different responses of the phosphorylated and non-phosphorylated proteins are not taken into account, and multiply-phosphorylated isoforms might not be detected because of their typically lower abundances and lower sensitivities. A label-free approach that did address this was done by Steen et al.[17] but it required the complicated determination of a "flyability ratio" to correct for ionization/detection differences between peptide and phosphopeptide before accurate quantitation of phosphorylation stoichiometry could be achieved.

Methods using phosphorylated standards, whether stable-isotope labeled, iTRAQ labeled, or unlabeled, take into account the variation in responses of the different peptides and phosphorylated forms, either by using synthetic phosphopeptides as internal standards [18-20] or by using synthetic peptides to create external calibration curves.[21] Generation of these phosphorylated standard peptides has been done by in-vitro phosphorylation of synthetic proteins [19] or by synthesizing isotopically-labeled reference standards, [18-21]. Monitoring of these phosphopeptides has been done by selected ion monitoring [17, 22, 23], and by MALDI using differential N-terminal labeling [24] or iTRAQ [5, 25] and MRM [18-21]. These techniques do take into account the differences in sensitivities of different isoforms – if they can be detected. Challenges in detecting low-sensitivity multiply-phosphorylated peptides still remain. Moreover, synthesis and quantitation of phosphopeptides is still difficult, even with the use of amino acid analysis and CE separation.

To overcome these issues, studies have been done using phosphatase to remove the phosphoryl groups. This increases the sensitivities of the peptides, and may enable the detection of previously undetectable multiply-phosphorylated peptides [23, 25, 26]. This technique has been used with both MALDI [13, 24, 25] and ESI [23, 26, 27]. Removing the phosphoryl groups has the advantage of facilitating quantitation – only the relative or absolute abundances of the non-phosphorylated isoforms need to be compared. The use of the phosphatase allows the determination of phosphorylation stoichiometry by observing a single peptide. Phosphatase treatment has been done with the additional use of a normalization technique to increase accuracy, such as iTRAQ labeling [25] or isotope labeling by methyl esterification [26]. Both methods, however, suffer from incomplete labeling (3% and 17%, respectively). Another labeling method by Previs et al [27] used phosphatase treatment at the protein level to avoid issues with variable trypsin cleavage close to sites of phosphorylation. The labeling chemistry, however, had an average efficiency of 87%, and for certain peptides as low as 38%. The QconCAT methodology of Johnson *et al*. [23] has combined isotopically-labeled peptides that act as specific standards for increased specificity and accuracy with the use of phosphatase treatment. This method requires the complex production of an isotope-labeled concatenated peptide and was demonstrated with the less specific detection by XIC.

We have developed a multiplexed, high-throughput, targeted method to determine the phosphorylation stoichiometry of selected phosphopeptides. Our assay, termed phosphatase-based phosphopeptide quantitation (PPQ) uses multiple reaction monitoring (MRM) mass spectrometry (MS) for detection and absolute quantification of the phosphorylation stoichiometry of phosphorylated peptides in complex biological samples. This is achieved through the use of enzymatic de-phosphorylation combined with specific and accurate peptide identification and quantification by MRM-MS detection[28], using stable-isotope-labeled standard (SIS) peptides. We have compared our new PPQ-MRM method (Figure 1) with the standard MRM method which uses phosphorylated SIS peptides.

The uniqueness of the PPQ-MRM method stems from the fact that we combine alkaline phosphatase, SIS peptides and detection by MRM. The method uses alkaline phosphatase to determine the proportion of phosphorylation (stoichiometry). The SIS peptides allow for increased accuracy of quantitation and increased specificity for the peptide of interest. Detecting the signal by MRM provides higher sensitivity and increased specificity compared to extracted ion chromatograms. None of the previous studies rigorously determined the limits of detection or accuracy of the determined phosphorylation stoichiometry or were compared to the direct MRM method with synthetic phosphopeptide standards.

Our approach is based on testing these two methods with mixtures of synthetic peptides and phosphopeptides, with different proportions of phosphorylation and in varied amounts,

spiked into a complex peptide background allowing for a very stringent determination of accuracy and reproducibility of the PPQ-MRM and direct-MRM methods. Signal transducing phosphoproteins are centrally involved in tumorigenesis, cancer progression, and drug susceptibility.[29, 30] Since breast cancer is often related to genetic defects resulting in aberrations in the function of the epidermal growth factor receptor (EGFR) [31-36] and estrogen receptor (ER) signaling pathways.[37-41] we chose, therefore, to use phosphopeptides from these breast cancer signaling pathways as model peptides for developing our PPQ-MRM method.

Materials & Methods

Reagents and chemicals

All reagents were American Chemical Society (ACS) grade or higher. All solvents used, including water, were LC/MS grade.

Peptide synthesis

Peptides, phosphopeptides, and internal standard (phospho)peptides containing stable-isotope coded amino acids ([$^{13}C_6$]Lys, [$^{13}C_6$, $^{15}N_4$]Arg, or [$^{13}C_6$ ^{15}N] Leu (98% isotopic enrichment)) were synthesized and purified as explained in Kuzyk et al. [42], and their actual concentrations and purity were determined by amino acid analysis (AAA) and capillary zone electrophoresis (CZE), respectively [42]. We targeted five unique amino acid sequences, one in the ERα protein, two in the HER2 protein, one for the RAF proteins (found in A-RAF, B-RAF and C-RAF), and one for ERK1 (Table 1). For each sequence, a non-phosphorylated peptide and a phosphopeptide were synthesized in their natural form, and in addition each was synthesized as a stable-isotope-labeled version to serve as a standard in the MRM analysis. Four (phospho)peptides were therefore made for each sequence.

MRM Q1/Q3 ion pair selection by nanoinfusion

MRM parameters, such as declustering potential (DP) voltage and collision energy (CE) voltage, were optimized for all peptides and phosphopeptides to obtain the maximum product ion signal intensity as explained in Kuzyk et al. 2009 [42, 43]

Cell culture and immunoprecipitation of ERα

The MCF-7 breast cancer cell line was grown in non-estrogen depleted medium to 80-90% confluence on 20 15 cm plates and ERα was immunoprecipitated using a rabbit anti-ERα antibody (HC-20) (Santa Cruz Biotechnology Inc. Santa Cruz, CA) as previously described, [44] with the exception that 0.03% CHAPS was used as the detergent in the IP buffer and ERα was eluted from beads using 5% acetic acid and subsequently lyophilized before digestion.

Tryptic digestion

An acetone protein extract from *E. coli* pellet was dissolved in 1.2% (w/v) sodium deoxycholate in 25 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA) at a 0.63 μg/μL protein concentration. Proteins were then reduced for 30 min at 60 °C with 5 mM tris(2-carboxyethyl)phosphine (TCEP, Thermo Scientific, IL, USA). The reduced sample was alkylated for 30 min at 37 °C in the dark with 10 mM iodoacetamide (Sigma-Aldrich, St. Louis, MO, USA). Modified, sequencing grade trypsin (Promega, Madison, WI) was added to the sample at a 50:1 substrate:enzyme ratio. The final concentrations in 25 mM ammonium bicarbonate were 0.5 μg/μL protein and 1% (w/v) sodium deoxycholate. Digestion was carried out for 16 h at 37 °C. The sample was then acidified by adding an equal volume of 1% (v/v) formic acid to stop digestion. The digest was concentrated by solid phase extraction using Waters Oasis reversed-phase 10 mg HLB cartridges (Waters,

Milford, MA) following the manufacturer's recommended protocol. The sample was eluted with 200 μL of 50% (v/v) acetonitrile, 0.1% (v/v) formic acid, and lyophilized to dryness. Peptides were then reconstituted in 0.1% formic acid to 5 μg/μL. For immunoprecipitated ERα protein from MCF-7 cells and for insect-cell line expressed ERα protein (20 pmol) (Sigma-Aldrich), the digestion procedure was as above, with the addition of the corresponding ERα standard peptides and phosphopeptides before the solid phase extraction step.

Preparation of synthetic peptide and phosphopeptide mixtures, PPQ (phosphatase-based phosphopeptide quantitation)-MRM and direct-MRM methods

Each synthesized peptide and phosphopeptide was made up to exactly a 10 pmol/μL concentration in 100 mM ammonium bicarbonate. The natural (unlabeled form) peptides and phosphopeptide were combined in proportions to create samples that contained a 0, 10, 20, 40, and 100% amount of each phosphopeptide. These samples were then split and half was used for the PPQ-MRM method and the other half was used for the direct-MRM method. To the PPQ-MRM designated samples, non-phosphorylated standard peptides were added for each corresponding peptide to equal the total amount of the natural peptide-phosphopeptide mixture (40 pmol). These samples were then serially diluted by 5-fold dilutions and subsequently mixed with an *E. coli* digest to be able to obtain on-column loadings of 1 pmol, 200 fmol, 40 fmol, and 8 fmol of each natural peptide-phosphopeptide mix and corresponding standards, in each case in a 1 μg amount of background peptides from *E. coli*. The same was done to the samples designated for the direct-MRM analysis except that phosphopeptide standards were added. The PPQ-MRM samples were then split into two, and half was dephosphorylated by adding alkaline phosphatase (calf intestine, EIA grade, Roche, Mannheim, Germany) at 0.64 U per μg of *E. coli* peptides (or per 1 pmol, 200 fmol, 40 fmol, or 8 fmol of peptide-phosphopeptide mix depending on the sample). An equivalent amount of 100 mM ammonium bicarbonate was added to the untreated half. Both samples were incubated for 2 hours at 37°C. The direct-MRM samples were not incubated. All the samples from the PPQ-MRM and direct-MRM methods were then acidified by addition of formic acid to 0.1% and bringing the volume to 1.0 ml. All samples were then desalted and concentrated by solid phase extraction using Waters Oasis reversed-phase 10 mg HLB cartridges following the manufacturer's recommended protocol. Samples were eluted with 200 μL of 50% (v/v) acetonitrile, 0.1% (v/v) formic acid. The eluted samples were frozen and lyophilized to dryness. Samples were reconstituted in 0.1% formic acid and 1 μL was injected for the nano-LC-MRM-MS analysis to give an on-column amount of 1000, 200, 40 or 8 fmol of total peptide-phosphopeptide mixture and corresponding (phospho)peptide standard, plus1 μg of *E. coli* background matrix peptides.

The ERα protein samples were treated as above except that only the corresponding standard (phospho)peptides were added to the samples post digestion at an equimolar amount to the predicted ERα protein concentration in the sample. For the direct-MRM samples both the phosphorylated and non-phosphorylated versions of the ERα peptide were added and for the PPQ-MRM sample only the non-phosphorylated version was added. The ERα samples were analyzed in duplicate.

LC-MRM-MS analysis of samples

An Eksigent NanoLC-1Dplus HPLC was used for the injection of samples (1 μL) onto a C18 Pepmap trap column (5 × 0.3 mm, 5 μm particles, Dionex/LC Packings) at 5 μL/min loading pump solvent (2% acetonitrile, 0.1% formic acid) for 3 min followed by separation on a reversed-phase capillary column (75 μm × 15 cm) packed in house using Magic C18AQ (5-μm diameter particles, 100-Å pore size; Michrom, Auburn, CA). Solvent A was 0.1% formic acid. Separations were performed using a flow rate of 300 nL/min with a 10-

min linear gradient from 0 to 24% solvent B (98% acetonitrile, 0.1% formic acid) followed by a 4-min linear gradient from 23 to 44% solvent B.

An Applied Biosystems/MDS Sciex 4000 QTRAP with a nanoelectrospray ionization source controlled by Analyst 1.5 software (Applied Biosystems) was used for all LC-MRM-MS analyses. All acquisition methods used the following parameters: 1900–2000-V ion spray voltage, a curtain gas setting of 25 p.s.i. (UHP nitrogen), a 150 °C interface heater temperature, a collision-activated dissociation pressure at 3.5×10^{-5} torr, and Q1 and Q3 set to unit resolution (0.6–0.8-Da full width at half-height). Uncoated fused silica emitter tips (20-μm inner diameter, 10-μm tip; New Objective, Woburn, MA) were used with 3–5-p.s.i. sheath gas and postcolumn, prespray addition of makeup solvent (80% isopropanol, 10% acetonitrile) at a flow rate of 100 nL/min using a PicoPlus 11 syringe pump (Harvard Apparatus). MRM acquisition methods were constructed with peptide-specific tuned declustering potential (DP) and collision energy (CE) voltages. A default collision cell exit potential of 23 V was used for all MRM ion pairs, with a target scan time below 2 s.

MRM data analysis

All MRM data was processed using MultiQuant 1.1 (Applied Biosystems) with the MQL algorithm for peak integration. Automatic peak detection using a 2 min retention time window, with “report largest peak” enabled and a 1-point Savitsky-Golay smooth with a peak-splitting factor of 2, was used. The default MultiQuant values for noise percentage and baseline subtraction window were used. All integrated peaks were manually inspected to ensure correct peak detection and accurate integration. All data was acquired using 3 MRM ion pairs per peptide. Reported data are derived from the quantifier MRM transition, with two qualifier transitions acting to verify retention times and reveal any signal interference. Qualifier ion pairs were used to detect the presence of interferences by ensuring that the relative signal intensity of ion pairs was consistent between the isotopically-labeled and endogenous forms of all peptides. For details of our method for selection of the MRM pairs and optimization of the tuning for each pair, see Kuzyk, *et al*. [43].

Calculations for percent phosphorylation

Peak area ratios were determined from the integrated peak areas for the natural peptide versus the peak area of the corresponding isotopically-labeled standard. For PPQ-MRM, the difference in the natural peptide signal between the untreated and phosphatase treated sample indicates the amount of phosphorylated peptide present in the sample (% phosphorylation = ((area ratio treated – area ratio untreated) / area ratio treated) × 100). This can be expressed as a ratio of phosphorylation (% phosphorylation) or as an absolute amount based on the known SIS concentration. To determine phosphorylation stoichiometry in the direct-MRM method *two* SIS peptides, the non-phosphorylated and the phosphorylated versions, need to be used in the analysis of the same sample. In this case, precisely-known amounts of *both* SIS peptides have to be present, and the two ratios of the natural targets to the standards can be used to determine the phosphorylation stoichiometry (% phosphorylation = (area ratio phosphorylated / (area ratio non-phosphorylated + area ratio phosphorylated)) × 100) . In the experiments reported here, the same mixtures of phosphopeptides were analyzed to allow a comparison of the two different methods.

Statistics

A Pearson Correlation two-tailed test was used to show significance of correlation between data sets.

Results and Discussion

In order to validate the PPQ-MRM method, five phosphopeptides from ERα, HER2, RAF, and ERK1 proteins were tested (Table 1). These were synthesized as natural and stable-isotope-labeled standards in both phosphorylated and non-phosphorylated forms, and were then added in different ratios and amounts to a 1 μg *E. coli* protein tryptic digest which acted as a complex background matrix. The total test-peptide amounts were kept at either 8, 40, 200 or 1000 fmol on column, while the phosphorylation ratio of each peptide was tested at 0, 10, 20, 40 or 100% phosphorylation. Three analytical replicates were performed on each mixture. The PPQ-MRM method was tested by comparing it to an MRM method that detected the phosphopeptides of interest directly (direct-MRM). An MRM method was developed so that all of these 20 peptides (10 targets, natural and labeled versions) could be compared in a single multiplexed LC-MS/MS analysis (Figure 2).

MRM acquisition methods were constructed using 3 MRM ion pairs per peptide using peptide-specific tuned declustering potential (DP) and collision energy (CE) voltages. Using our synthetic peptides and phosphopeptides, fragment ions were experimentally selected to obtain the maximum sensitivity for each target peptide. Additionally, DP and CE voltages were optimized for each peptide target and fragment ion, respectively, to maximize signal strength. Despite this (phospho)-peptide specific tuning, however, the signal strength for the phosphopeptides tested was always lower than that for the corresponding non-phosphorylated peptide (Figure 2). This supports our hypothesis that looking at the de-phosphorylated peptide would maximize detection sensitivity and quantitation accuracy.

Figure 3 shows an example of the MRM results for the HER2 Y1248 phosphopeptide at two different phosphorylation levels (20 and 60%), as determined by the PPQ-MRM technique and the direct-MRM approach. In each case, stable-isotope-labeled standard (SIS) peptides are included for specific detection and accurate quantitation. In the PPQ-MRM method this is a labeled version of the non-phosphorylated form of the target; in the direct-MRM method this is an isotopically-labeled version of the phosphorylated and non-phosphorylated form of the target. This indicates how the PPQ-MRM method requires a single SIS peptide for phosphorylation stoichiometry determination but two analyses, and how the direct-MRM method requires one analyses but two SIS peptides. With a single phosphorylated SIS, the direct-MRM method can only determine relative phosphorylation differences.

The accuracy and reproducibility of these assays, as well as the limit of quantitation for the phosphopeptides, were assessed. A comparison between the actual % phosphorylation and the observed % phosphorylation, are shown in Figure 4 for peptides at different phosphorylation ratios (0, 10, 20, 40 or 100% phosphorylation) and four different total peptide amounts (8, 40, 200, or 1000 fmol on column) for each method. Three analytical replicates are shown for each combination.

As can be seen from Figure 4, the results obtained for the singly-phosphorylated phosphopeptides using direct-MRM and PPQ-MRM are nearly identical (correlation >0.992, $p < 4.3e-13$) at the higher total peptide amounts (1 pmol and 200 fmol). At the lower peptide amounts (40 and 8 fmol), there is a larger difference (correlation >0.913, $p < 2.0e-6$) between the two methods, and both are less accurate as discussed below. In general, for both methods the accuracy is higher at the higher total peptide amounts and at the higher phosphorylation ratios. A stoichiometric (100%) phosphorylation can be determined accurately (<±4% error) at the lowest peptide level tested (8 fmol), with lower phosphorylation ratios becoming less accurate.

In addition, the accuracy is peptide dependent. This can be seen for the two HER2 peptides that are phosphorylated on tyrosine. Using the PPQ-MRM method the phosphorylation

ratios (20 to 100% phosphorylation) can be accurately (<±20 % error) determined at the 8 fmol level for the HER2 Y877 peptide but only at the 200 fmol level and higher for the HER2 Y1248 peptide. This appears to be related to the MRM signal intensity of theses peptides (see Figure 2). For the doubly-phosphorylated ERK1 peptide, both methods appear to overestimate the level of phosphorylation (30-300% depending on peptide level and test phosphorylation level). This was attributed to be a problem with the determining the concentration of the doubly-phosphorylated peptide using AAA and CZE rather than the PPQ-MRM or direct-MRM methods. Despite the fact that the phosphopeptide concentration was adjusted based on AAA and CZE values, the MRM peak areas gave an overestimation of ~40% from the expected amounts of both the labeled and unlabeled versions of the ERK1 phosphopeptide (data not shown). In contrast, the corresponding non-phosphorylated version of this peptide gave the expected MRM peak areas. We also tried to synthesize the ERK2 phosphopeptide which had four phosphorylation sites. The labeled and unlabeled versions of the phosphopeptide could not be successfully synthesized or detected despite having good AAA values (non-detectable by CZE), although the corresponding non-phosphorylated peptide showed a strong MRM signal (data not shown). These findings demonstrate the challenges and difficulties involved with the use of phosphopeptides as internal standards, which seem to have problems with stability and MS detection, or possibly even with synthesis and AAA/CZE concentration/purity determination. These problems with the accurate quantitation of multiply-phosphorylated peptide *standards*, provide further support for the PPQ-MRM method, which only uses the non-phosphorylated form of the peptide as an internal standard, despite the methods inability to provide direct information on the position of the phosphorylation site in peptides with multiple potential phosphorylation sites.

A comparison of accuracies for the two methods for determining the actual percent of phosphorylation is shown in Figure 5. The accuracy is shown for the PPQ-MRM method (red dots) and the direct-MRM (blue squares) method for determining phosphorylation stoichiometry in all of the singly-phosphorylated peptides at the different total peptide amounts (1 pmol, 200 fmol, 40 fmol, and 8 fmol). These graphs show the percent accuracy for all the replicate determinations at the different phosphorylation ratios tested. The ± 20% accuracy region is shaded in blue. As expected, the accuracy for both methods is higher at higher total peptide amounts (an average of 89% at 1 pmol vs. 60% at 8 fmol) and when the ratio of phosphorylation is greater (an average of 84% accuracy at 40% phosphorylation vs. 54% accuracy at 10% phosphorylation). For both methods, the accuracy was highly dependent on the particular peptide studied as indicated earlier. However, looking at all the peptides together these results show that the PPQ-MRM method is more accurate than the “classical” direct-MRM method for determining phosphorylation stoichiometry at the higher levels (an average of 93% vs. 85% at 1 pmol respectively and an average of 86% vs. 85% at 200 fmol respectively), and less accurate at the lower concentrations but is still close to the direct MRM method (an average of 71% vs. 84% for 40 fmol respectively, and an average of 56% vs 63% at 8 fmol).

To determine the lower limit of quantitation (LLOQ, defined as a reproducible measurement with better than 20% precision [45]) for each method, the reproducibility of each method was also considered. These results are summarized in Table 2. This table indicates the minimum phosphorylation stoichiometry (% phosphorylation) that can be determined with an accuracy of ± 20% and a CV of <20% between three replicate analyses of the same sample, for each peptide and for each total peptide level (8, 40, 200 fmol, or 1 pmol). The first number indicates the minimum phosphorylation stoichiometry that can be determined with the actual observed phosphorylation level in brackets.

The PPQ-MRM method was able to accurately and reproducibly (error ±20% and CVs <20%) detect as little as a 10% (20 fmol) level of phosphorylation in 200 fmol of peptide in

1-μg complex sample, while the direct-MRM method could achieve this at the 40 fmol level. At the lowest level (8 fmol total peptide), the PPQ-MRM method was able to accurately and reproducibly detect a 20% phosphorylation stoichiometry of two peptides, HER2 Y877 and RAF. This is equivalent to 1.6 fmol of phosphorylated peptide in a 1 μg of complex peptide background. At the same 8 fmol total peptide level, the direct-MRM method could, at best, only accurately and reproducibly determine a 40% phosphorylation stoichiometry.

The numbers in red in Table 2 indicate the lowest % phosphorylation at the lowest total-peptide level, which can be accurately and reproducibly determined by each method for each peptide (*i.e.*, the LLOQ). As demonstrated in this Table, the PPQ-MRM method performs comparably to the direct-MRM method. Looking at the singly-phosphorylated peptides, the PPQ-MRM has a lower LLOQ for the HER2-Y877 and RAF peptides, and the same LLOQ for the ERα peptide, while the direct-MRM method has a lower LLOQ for the HER2-Y1248 peptide. On average, the PPQ-MRM method has a significantly lower LLOQ than the direct-MRM method at the higher total peptide amounts (with an average LLOQ of 15% *vs*. 40% at 1 pmol, and 22.5% *vs*. 40% at 200 fmol) and a similar LLOQ at the lower amounts (average LLOQ of 50% *vs*. 42.5% at 40 fmol respectively and 45% *vs*. 55% at 8 fmol).

For the HER2 Y877 phosphopeptide, the PPQ-MRM method performs better at all peptide levels than the direct-MRM method, but the opposite is true for the HER2 Y1248 phosphopeptide at the 8 and 40 fmol. Both methods are comparable in performance for the ERα and RAF peptides. As mentioned above, for the doubly phosphorylated ERK1 both methods give an overestimation of the true ratios due to an underestimation of the AAA-based phosphopeptide quantitation. The direct-MRM method appears to give less of an overestimation, probably due to the fact that the concentrations of both the natural and standard phosphopeptides were equally underestimated, while the PPQ-MRM method relied on a non-phosphorylated standard.

To demonstrate that the PPQ-MRM methodology is applicable to “real life” protein containing samples, we performed an analysis of the S154 phosphorylation site-containing peptide on two samples known to contain ERα protein. The S154 site is a recently-discovered phosphorylation site in ERα with new potential as a relevant target in ER-positive breast cancer,[46] and we set out to test its phosphorylation state in the MCF-7 breast cancer cell line. Both the PPQ-MRM and direct-MRM methods were used to analyze a sample of an immunoprecipitated (IP) ERα protein from un-induced MCF-7 cells, and a sample of an ERα protein expressed in an insect cell line that is known to produce phosphorylated protein [47].

The ERα protein obtained from the un-induced MCF-7 cells was shown *not* to be phosphorylated at the S154 site by both the direct-MRM and the PPQ-MRM methods (Figure 6). Moreover, both methods are in close agreement that the protein is present at an amount of ~5 fmol (on column) in the IP sample (Table 3). Analysis of the ERα protein expressed in an insect cell line revealed a low level of phosphorylation at the S154 residue by both methods (~9%). Both methods are also in agreement that the total protein is present at ~970 fmol on column. General serine phosphorylation of recombinant ERα protein from insect cells lines has been observed by Western blots [47], with specific phosphorylations at S167 and Y537 being reported [48], This could make our observation of a phosphorylation at S154 in these cells a novel finding. In any event, these experiments confirm that the PPQ-MRM method can successfully be used for the analysis of human breast cancer cells, being capable of proving a *lack* of phosphorylation on a peptide from a signaling protein as well as indicating a level of phosphorylation stoichiometry starting from a whole protein.

The PPQ-MRM method is best suited for phosphopeptides with a well-defined single phosphorylation site. If multiple phosphorylation sites are possible or are not well defined, the phosphorylation ratio can be defined as "% of phosphorylated peptide" and cannot be attributed to a specific site. This would be disadvantageous in studies where a kinase site with multiple phosphorylation residues within a single tryptic peptide is being analyzed, and information on differential positional phosphorylation is important. Closely-spaced phosphorylation sites occur often in signaling proteins and, in this case, the direct-MRM method would be a better approach, giving information on specific residues as was done for the Cyclin dependent kinase (Cdk) regulatory site T14-Y15 in the study by Mayya et al.[20] However, it should be pointed out that the PPQ-MRM method allows for eliminating all of the potential phosphorylation sites within a peptide in a single experiment.

Although the PPQ-MRM method might not provide direct positional information for the phosphoryl group, the process of dephosphorylating multiply phosphorylated peptides increases the likelihood of observing a phosphorylation event that may otherwise be missed using standard MS techniques which usually exhibit lower signal intensities for multiply-phosphorylated peptides. Additionally, recent evidence [6, 7] and our own observations with synthetic phosphopeptides show that phosphate group rearrangements easily occur upon CID making MS/MS data that specifies phosphorylation location more dubious and makes phosphorylation stoichiometry data for a peptide despite its lack of positional information significant.

Using our model phosphopeptides and alkaline phosphatase treatment, we observed a 99.3% to 100% dephosphorylation efficiency (depending on the peptide) based on the MRM signal detected for the phosphopeptide after phosphatase treatment, even at a level of 1 pmol per peptide in a 1 μg complex peptide background. It is possible, of course, that other phosphopeptides could have lower dephosphorylation efficiencies, but these efficiencies could be determined by tests on synthetic target phosphopeptides, and the calculation to determine the original phosphoprotein concentration could be modified to take such efficiencies into account. Alternative or multiple phosphatases, as lambda or shrimp alkaline phosphatase, could be used to obtain improved dephosphorylation if necessary. However, in the PPQ-MRM method, the dephosphorylation is done at the peptide level, not at the protein level, and all three phosphoamino acids were observed to be efficiently dephosphorylated. Thus, the problem of ineffective phosphate removal may be less of a problem than it is at the protein level.[26, 27] However, dephosphorylation at the protein level, with subsequent tryptic digestion, might be preferred in cases where the tryptic cleavage site for a peptide of interest is close to the phosphorylation site and could influence tryptic cleavage.[27]

Target signaling proteins are likely to be present in low amounts in the cell lysate. For this reason, enrichment procedures have been used such as TiO_2 chromatography to enrich samples for phosphopeptides before MS analysis. The PPQ-MRM method and other methods that rely on the direct determination of % phosphorylation rely on obtaining both the phosphorylated and the non-phosphorylated forms of the peptide of interest out of the sample at equal efficiencies and therefore enrichment techniques specific for phosphopeptides cannot be used. Antibodies, however, specific to the phosphoprotein or (phospho)-peptide that target epitope sites independent of the phosphorylation state, could be used to enrich the sample in these low-abundance target peptide or proteins.[49] As was done by Mayya et al [20] such antibodies could also add specificity to the detection as is the case for RAF in our study where one peptide represents multiple phosphoproteins that share this same common motif or active site. Mayya et al. [20] were able to distinguish stoichiometry on different cyclin-dependent kinases (Cdk 1, 2 and 3) that shared an identical phosphopeptide in their inhibitory site through the use of Cdk specific antibodies in their immunoprecipitations.

Conclusions

The PPQ-MRM method was able to accurately and reproducibly detect a 10% phosphorylation level in 200 fmol of peptide in 1-μg complex sample (an *E. coli* digest) and a 20% phosphorylation level in 8 fmol of peptide. In comparison, the direct-MRM detected the lowest % phosphorylation (10%) in 40 fmol of peptide and a 40% phosphorylation level in 8 fmol of peptide. The sensitivity and reproducibility of the PPQ-MRM method depends *only* on the sensitivity of the unphosphorylated peptide, which is usually significantly higher than the sensitivity of the phosphorylated peptide. Moreover, non-phosphorylated peptide standards are more stable than phosphopeptides, and are easier to synthesize and accurately quantitate.

One additional advantage is that only one peptide standard is required per phosphopeptide, in contrast to the direct-MRM method which requires a SIS peptide for each phosphorylated isoform. MRM-based assays can therefore be more readily multiplexed to allow the detection of a large number of peptides (representing a large number of phosphoproteins) in a single analysis. This method is analogous to running multiple western blots on a sample using highly-specific anti-phosphopeptide antibodies, but with the additional benefit of providing this phosphorylation-stoichiometry information at very high throughput. Analyses of samples containing ERα protein revealed that the PPQ-MRM is capable of determining phosphorylation stoichiometry in proteins from cell lines, and is in good agreement with determinations obtained using the direct-MRM approach in terms of phosphorylation and total protein amount.

Thus, the PPQ-MRM method should allow the high-throughput profiling of entire signaling pathways at high quantitation accuracy and sensitivity. This will allow the creation of detailed profiles or ‘maps’ of signaling networks, including, the EGFR and ER signaling networks in breast cancer tumor samples.

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Abbreviations

MRM	multiple reaction monitoring
PPQ	phosphatase-based phosphopeptide quantitation
MS	mass spectrometry
CID	collision-induced dissociation
LLOQ	lower limit of quantitation
ESI	electrospray ionization
MALDI	matrix-assisted laser desorption ionization
MS/MS	tandem mass spectrometry
ER	estrogen receptor
EGFR	epidermal growth factor receptor

HER2	Receptor tyrosine-protein kinase erbB-2
RAF	RAF proto-oncogene serine/threonine-protein kinase
ERK1	Mitogen-activated protein kinase 3
XIC	extracted ion chromatogram
SIS	stable isotope-coded standard
AAA	amino acid analysis
CZE	capillary zone electrophoresis
DP	declustering potential voltage
CE	collision energy voltage

References

(1). Mann M, Ong SE, Gronborg M, Steen H, Jensen ON, Pandey A. Trends Biotechnol 2002;20:261–268. [PubMed: 12007495]

(2). Johnson LN, Lewis RJ. Chemical reviews 2001;101:2209–2242. [PubMed: 11749371]

(3). Borchers CH, Thapar R, Petrotchenko EV, Torres M, Speir JP, Easterling M, Dominski Z, Marzluff WF. Proceedings of the National Academy of Sciences of the United States of America 2006;103:3094–3099. [PubMed: 16492733]

(4). Trinidad JC, Specht CG, Thalhammer A, Schoepfer R, Burlingame AL. Molecular & Cellular Proteomics 2006;5:914–922. [PubMed: 16452087]

(5). Munton RP, Tweedie-Cullen R, Livingstone-Zatchej M, Weinandy F, Waidelich M, Longo D, Gehrig P, Potthast F, Rutishauser D, Gerrits B, Panse C, Schlapbach R, Mansuy IM. Molecular & Cellular Proteomics 2007;6:283–293. [PubMed: 17114649]

(6). Palumbo AM, Reid GE. Anal. Chem 2008;80:9735–9747. [PubMed: 19012417]

(7). Palumbo AM, Tepe JJ, Reid GE. J. Proteome Res 2008;7:771–779. [PubMed: 18181561]

(8). Liao PC, Leykam J, Andrews PC, Gage DA, Allison J. Anal. Biochem 1994;219:9–20. [PubMed: 8059960]

(9). Stensballe A, Andersen S, Jensen ON. Proteomics 2001;1:207–222. [PubMed: 11680868]

(10). Zhou W, Merrick BA, Khaledi MG, Tomer KB. J. Amer. Soc. Mass Spectrom 2000;11:273–282. [PubMed: 10757163]

(11). Hsu JL, Chou MK, Liang SS, Huang SY, Wu CJ, Shi FK, Chen SH. Electrophoresis 2004;25:3840–3847. [PubMed: 15565681]

(12). Hirschberg D, Jagerbrink T, Samskog J, Gustafsson M, Stahlberg M, Alvelius G, Husman B, Carlquist M, Jornvall H, Bergman T. Anal Chem 2004;76:5864–5871. [PubMed: 15456308]

(13). Torres MP, Marzluff WF, Borchers CH. J. Proteome Res 2005;4:1628–1635. (2005), 1628-1635. [PubMed: 16212415]

(14). Ficarro SB, McCleland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM. Nat Biotechnol 2002;20:301–305. [PubMed: 11875433]

(15). Ruse CI, Willard B, Jin JP, Haas T, Kinter M, Bond M. Anal. Chem 2002;74:1658–1664. [PubMed: 12033257]

(16). Parker CE, Warren MR, Loiselle DR, Dicheva NN, Scarlett CO, Borchers CH. Methods Mol Biol 2005;301:117–151. [PubMed: 15917630]

(17). Steen H, Jebanathirajah JA, Springer M, Kirschner MW. PNAS 2005;102:3948–3953. [PubMed: 15741271]

(18). Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. Proc Natl Acad Sci U S A 2003;100:6940–6945. [PubMed: 12771378]

(19). Ciccimaro E, Hanks SK, Yu KH, Blair IA. Anal. Chem 2009;81:2009.

(20). Mayya V, Rezual K, Wu L, Fong MB, Han DK. Molecular & Cellular Proteomics 2006;5:1146–1157. [PubMed: 16546994]

(21). Glinski M, Weckwerth W. Molecular & cellular proteomics : MCP 2005;4:1614–1625.

(22). Ruse CI, Willard B, Jin JP, Haas T, Kinter M, Bond M. Anal.Chem 2002;74:1658–1664. [PubMed: 12033257]

(23). Johnson H, Eyers CE, Eyers PA, Beynon RJ, Gaskell SJ. J Am Soc Mass Spectrom 2009;20:2211–2220. [PubMed: 19783156]

(24). Zhang X, Jin QK, Carr SA, Annan RS. Rapid Communications in Mass Spectrometry 2002;16:2325–2332. [PubMed: 12478578]

(25). Pflieger D, Junger MA, Muller M, Rinner O, Lee H, Gehrig PM, Gstaiger M, Aebersold R. Molecular & Cellular Proteomics 2008;7:326–346. [PubMed: 17956857]

(26). Hegeman AD, Harms AC, Sussman MR, Bunner AE, Harper JF. J Am Soc Mass Spectrom 2004;15:647–653. [PubMed: 15121193]

(27). Previs MJ, VanBuren P, Begin KJ, Vigoreaux JO, LeWinter MM, Matthews DE. Analytical Chemistry 2008;80:5864–5872. [PubMed: 18605695]

(28). Wolf-Yadlin A, Hautaniemi S, Lauffenburger DA, White FM. Proceedings of the National Academy of Science U S A 2007;104:5860–5865.

(29). Wood LD, Parsons DW, Jones S, Lin J, Sjoeblom T, Leary RJ, Shen D, Boca SM, Barber T, Ptak J, Silliman N, Szabo S, Dezso Z, Ustyanksky V, Nikolskaya T, Nikolsky Y, Karchin R, Wilson PA, Kaminker JS, Zhang Z, Croshaw R, Willis J, Dawson D, Shipitsin M, Willson JKV, Sukumar S, Polyak K, Park BH, Pethiyagoda CL, Pant PVK, Ballinger DG, Sparks AB, Hartigan J, Smith DR, Suh E, Papadopoulos N, Buckhaults P, Markowitz SD, Parmigiani G, Kinzler KW, Velculescu VE, Vogelstein B, Sjoeblom T, Beerenwinkel N, Vogelstein B, Kinzler KW. Science Nov 16;2007 318(5853):1108–13. 318, 1108-1113. [PubMed: 17932254]

(30). Jones S, Zhang X, Parsons DW, Lin JC-H, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong S-M, Fu B, Lin M-T, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. Science 2008;321:1801–1806. [PubMed: 18772397]

(31). Magkou C, Nakopoulou L, Zoubouli C, Karali K, Theohari I, Bakarakos P, Giannopoulou I. Breast cancer research 2008;10:R49. [PubMed: 18522728]

(32). Guo L, Abraham J, Flynn DC, Castranova V, Shi X, Qian Y. Open Clinical Cancer Journal 2008;2:18–31.

(33). Kallergi G, Agelaki S, Kalykaki A, Stournaras C, Mavroudis D, Georgoulias V. Breast cancer research 2008;10:R80. [PubMed: 18822183]

(34). Helms MW, Kemming D, Contag CH, Pospisil H, Bartkowiak K, Wang A, Chang S-Y, Buerger H, Brandt BH. Cancer Research 2009;69:5049–5056. [PubMed: 19491269]

(35). Nieto Y, Nawaz F, Jones RB, Shpall EJ, Nawaz S. Journal of clinical oncology 2007;25:4405–4413. [PubMed: 17906204]

(36). Tong J, Taylor P, Peterman SM, Prakash A, Moran MF. Molecular and Cellular Proteomics 2009;8:2131–2144. [PubMed: 19531499]

(37). Zhang D, Wong LL, Koay ESC. Molecular Cancer 2007;6:52. [PubMed: 17697330]

(38). Yamashita H, Nishio M, Kobayashi S, Ando Y, Sugiura H, Zhang Z, Hamaguchi M, Mita K, Fujii Y, Iwase H. Breast cancer research 2005;7:R753–R764. [PubMed: 16168121]

(39). Valdehita A, Bajo AM, Schally AV, Varga JL, Carmena MJ, Prieto JC. Molecular and Cellular Endocrinology 2009;302:41–48. [PubMed: 19101605]

(40). Frogne T, Laenkholm A-V, Lyng MB, Henriksen KL, Lykkesfeldt AE. Breast cancer research 2009;11:R11. [PubMed: 19239686]

(41). Wong LL, Chang CF, Koay ESC, Zhang D. International Journal of Oncology 2009;34:1291–1301. [PubMed: 19360341]

(42). Kuzyk M, Smith D, Yang J, Cross T, Jackson A, Hardie D, Anderson L, Borchers C. Molecular and Cellular Proteomics 2009;8:1860–1877. [PubMed: 19411661]

(43). Kuzyk MA, Parker a. C. E. Borchers CH. Methods in Molecular Biology. 2009 in press.

(44). Weitsman GE, Li L, Skliris G, Davie JR, Ung K, Niu Y, Curtis-Snell L, Tomes L, Watson PH, Murphy LC. Cancer Research 2006;66:10162–10170. [PubMed: 17047081]

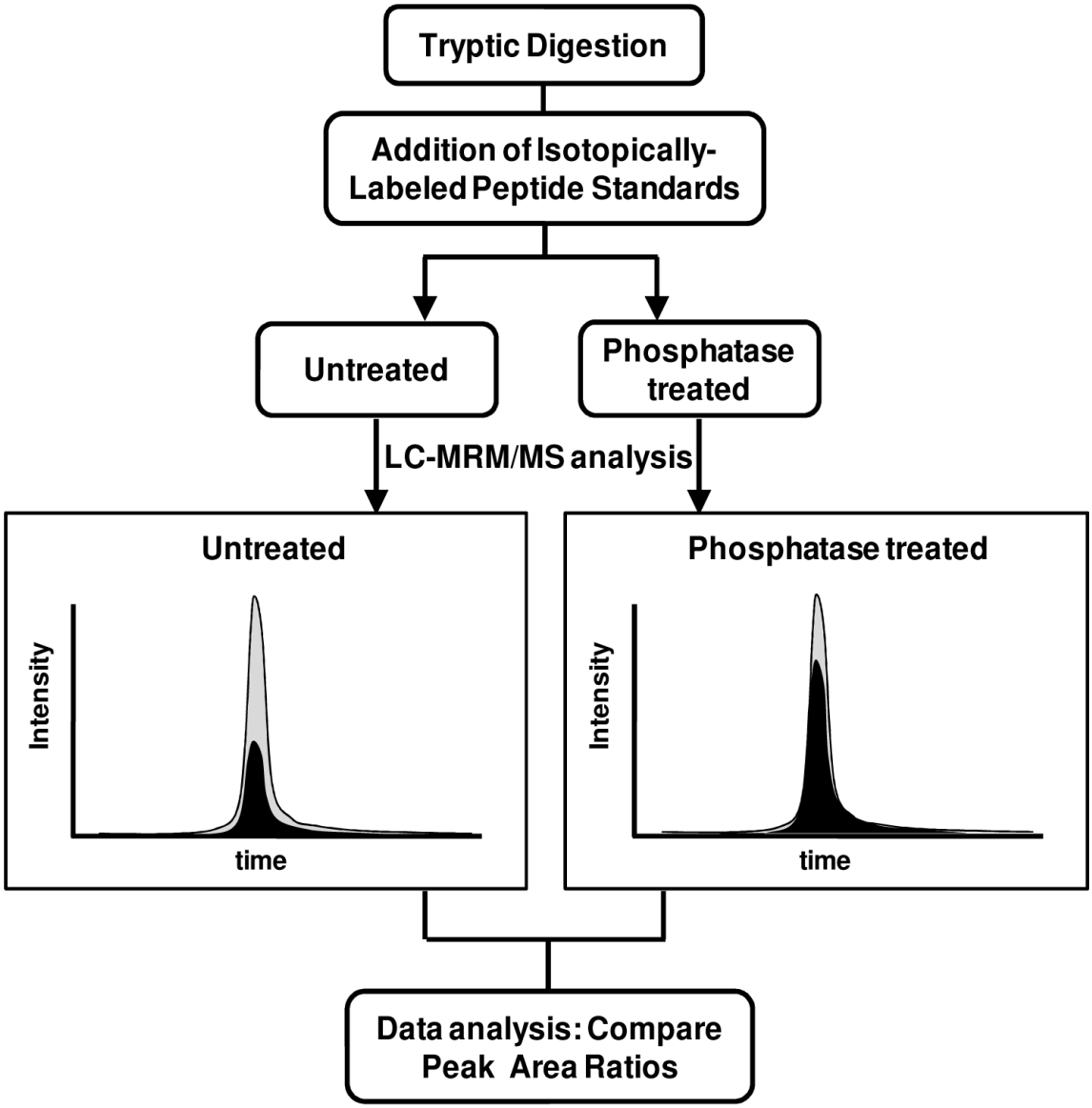
(45). Arinbruster DA, Tillman MD, Hubbs LM. Clinical Chemistry 1994;40:1233–1238. [PubMed: 8013092]

(46). Britton DJ, Scott GK, Schilling B, Atsriku C, Held JM, Gibson BW, Benz CC, Baldwin MA. Journal of the American Society for Mass Spectrometry 2008;19:729–740. [PubMed: 18367407]

(47). Murphy, LC. 2010. unpublished data

(48). Arnold SF, Obourn JD, Jaffe H, Notides AC. Molecular Endocrinology 1994;8:1208–1214. [PubMed: 7838153]

(49). Shah B, Reid JD, Kuzyk MA, Parker CE, Borchers CH. Methods in Molecular Biology. 2010



$$\% \text{ Phosphorylation} = \frac{\text{Area ratio treated} - \text{Area ratio untreated}}{\text{Area ratio treated}} \times 100$$

Figure 1. Experimental strategy of phosphatase-based phosphopeptide quantitation (PPQ) by MRM

Proteins are digested with trypsin and isotope-labeled standard peptides (non-phosphorylated forms of target) are added (light gray) to increase accuracy of quantitation and specificity of detection. The sample is split into two and buffer-only or phosphatase treated. Two LC-MRM-MS analyses are then performed. Phosphatase treatment will increase the signal of the natural peak (dark) if phosphorylation is present. Peak Area Ratios are then determined from the area of the natural peak (dark) versus the area of the internal standard (light gray). Phosphorylation stoichiometry can then be determined by comparing

the Peak Area Ratios from the untreated and phosphatase treated samples as indicated in the boxed formula (assuming 100% dephosphorylation efficiency).

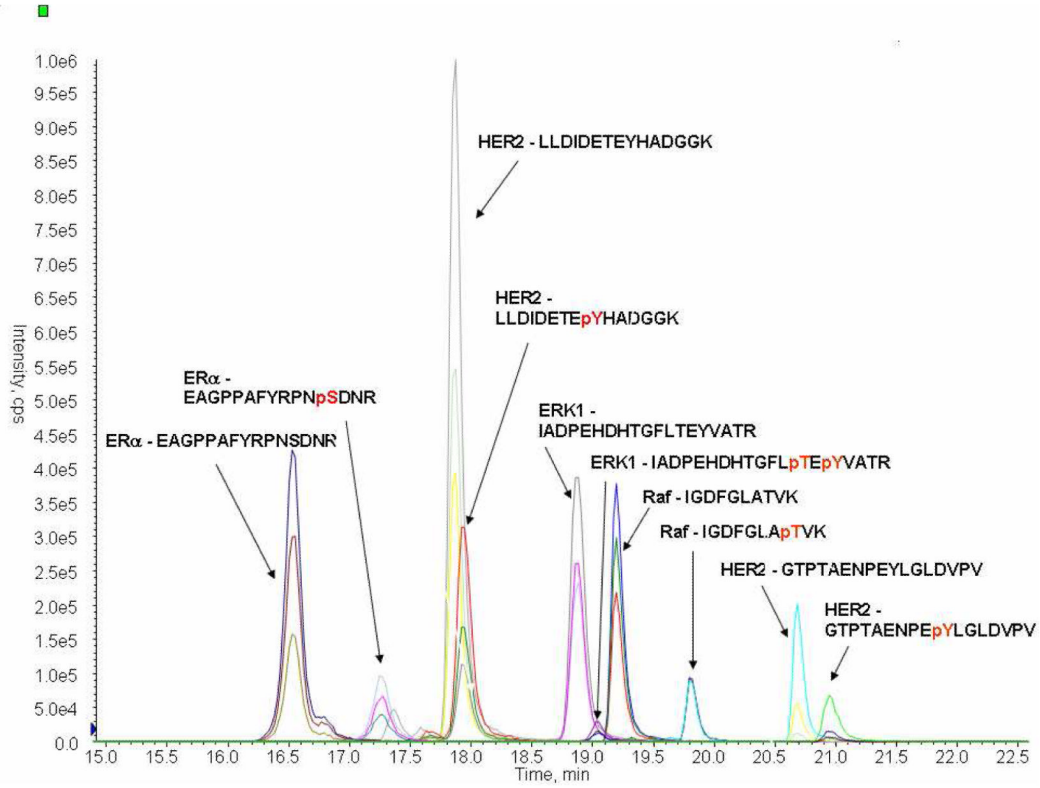


Figure 2. An LC-MRM/MS chromatogram showing 1 pmol of each peptide and corresponding phosphopeptide used in the study

Three MRM transitions producing the highest signals were used for each target and are shown by the overlapping peaks. Note that the non-phosphorylated versions of each peptide always produce higher-intensity signals.

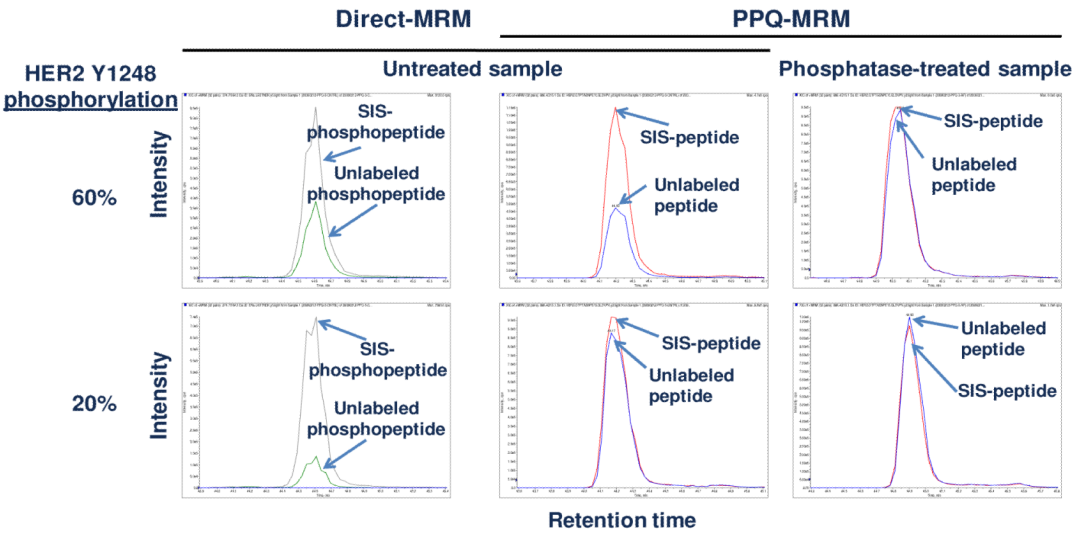


Figure 3. An example of LC-MRM/MS traces showing the concept of the PPQ-MRM and direct-MRM methods

The phosphorylated HER2 peptide was mixed with its non-phosphorylated form in two samples, and phosphorylation was quantitated using either the PPQ-MRM method or by direct-MRM. For example, for 60% phosphorylation stoichiometry, 6 parts of phosphorylated HER2 peptide was mixed with 4 parts of non-phosphorylated peptide and added to an *E. coli* digest to give a total peptide load of 1 pmol on column in 1 μg of digest. The stable-isotope-labeled standard (SIS) peptides were added at 1 pmol on column. The PPQ-MRM method shown by four chromatograms on the right requires two sample analyses of the phosphatase treated and untreated samples, detecting only the non-phosphorylated peptide with a single corresponding SIS peptide. The direct-MRM method shown by four chromatograms on the left requires a single analysis (of an untreated sample) detecting the non-phosphorylated and phosphorylated peptides with two corresponding SIS peptides.

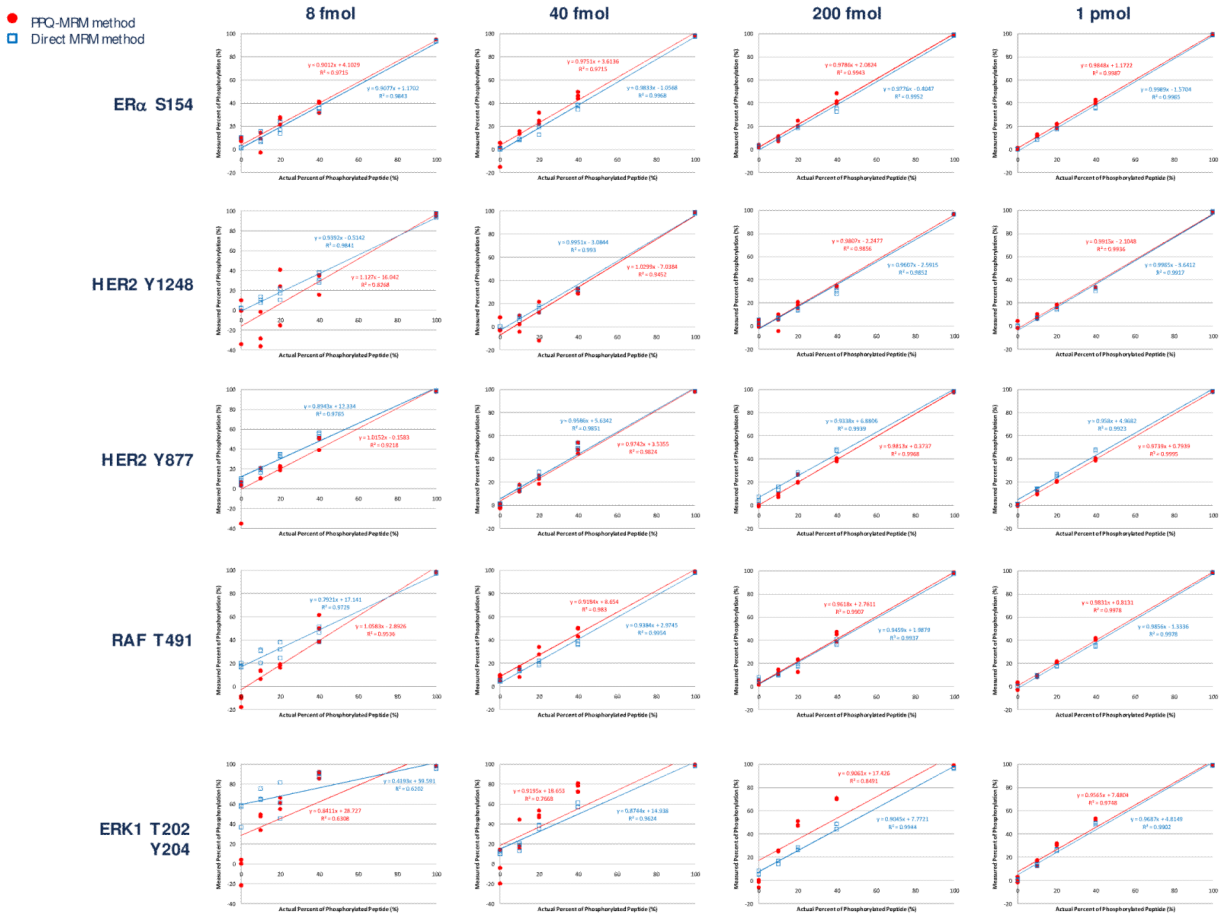


Figure 4. Determination of phosphorylation stoichiometry by PPQ-MRM and direct-MRM methods in HER2, ERα, RAF and ERK1 peptides

Synthetic peptides were mixed at different ratios (0, 10, 20, 40 or 100% phosphorylation stoichiometry) for a total amount of 8, 40, 200, or 1000 fmol on column in a 1 μg *E. coli* sample. Three analytical replicates were performed for each combination. Theoretical % phosphorylation is indicated on the x-axis with the determined % phosphorylation on the y-axis. Quantitation obtained with the PPQ-MRM method is indicated in red with the direct-MRM method indicated in blue. Formulas indicate a best fit linear curve of the data.

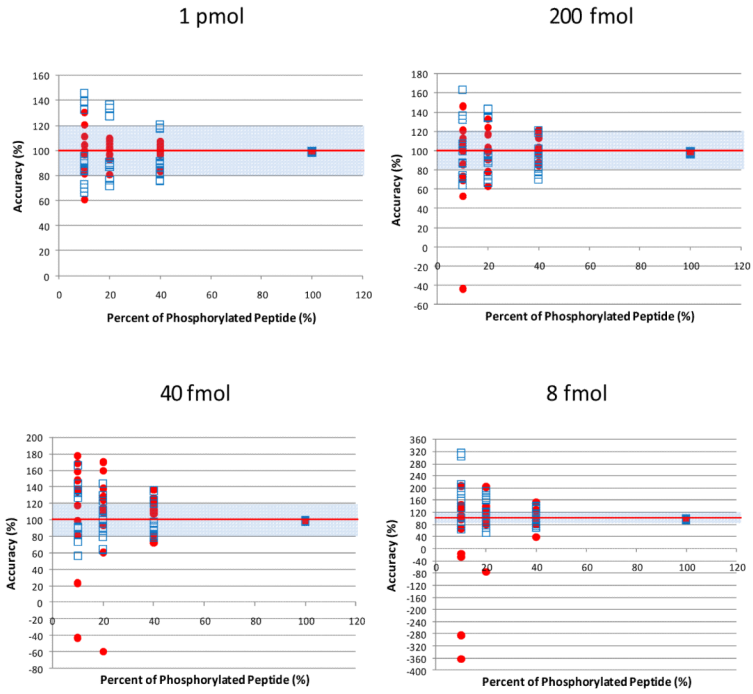


Figure 5. Accuracy of the PPQ-MRM method versus the direct-MRM method in determining phosphorylation stoichiometry

Graphs show the percent accuracy for the determination of correct phosphorylation stoichiometry for all the singly-phosphorylated peptides at different phosphorylation ratios and total peptide amounts of 8 fmol, 40 fmol, 200 fmol, or 1 pmol in triplicate analyses. The PPQ-MRM accuracies are indicated in red and those for the direct-MRM method are shown in blue. The area of accuracy between 80-120% is shaded in blue.

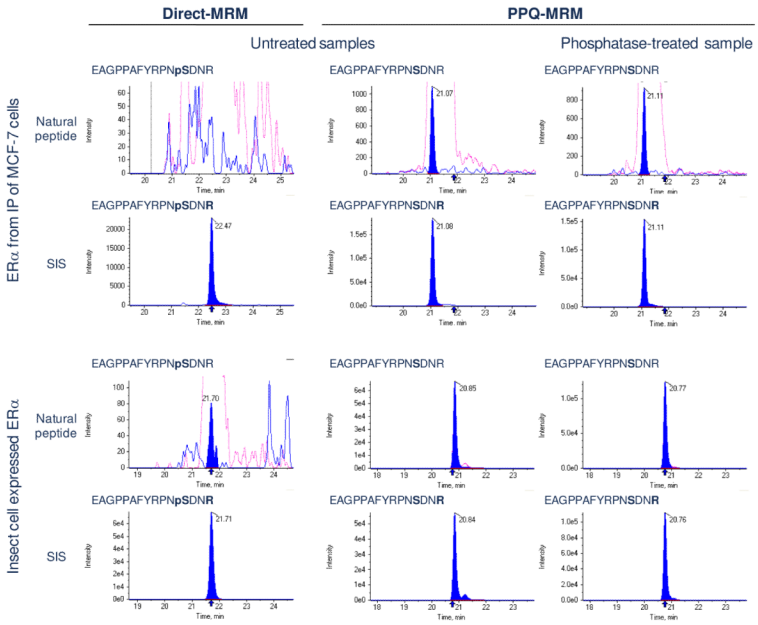


Figure 6. Analyses of ERα protein containing samples using the PPQ-MRM and direct-MRM methods

Two samples, one of an immunoprecipitated ERα protein from an un-induced MCF-7 breast cancer cell line, and one of an ERα protein expressed in an insect cell line were analyzed in duplicate using the PPQ-MRM and direct-MRM methods for the S154 phosphorylation site-containing peptide. Representative chromatograms of a single MRM transition (doubly positive charged y_{11}) are shown for the natural and SIS (phospho)peptide EAGPPAFYRPNSDNR. The target peptide is shown in solid blue and the co-eluting standard peptide is shown by the overlaid red line.

Table 1
Target phosphopeptides used to test the PPQ-MRM and direct-MRM methods

Four (phospho)peptides were made for each sequence shown: a non-phosphorylated peptide and a phosphopeptide were synthesized in their natural form, and each was also synthesized as a stable-isotope-labeled version to serve as a standard in the MRM analysis. The phosphorylated amino acids in the phosphopeptides are shown in bold and the stable-isotope-labeled amino acids in the standards are indicated with an asterisk (*). Full protein names and UniProt accession numbers for the target proteins are shown in parentheses.

Target Protein	Phosphorylation site	Detected peptide
ERα (Estradiol receptor alpha, P03372)	S154	143-EAGPPAFYRPNSDNR*-157
HER2 (Receptor tyrosine-protein kinase erbB-2, P04626)	Y1248	1239-GTPTAENPEYLGL*DVPV-1255
HER2 (Receptor tyrosine-protein kinase erbB-2, P04626)	Y877	869-LLDIDETEYHADGGK*-883
RAF (proto-oncogene serine/threonine-protein kinases, P04049, P15056, P10398)	T491	484-IGDFGLA**T**VK*-493
ERK1 (Mitogen-activated protein kinase 3, P27361)	T202, Y204	190-IADPEHDHTGFL**T**E**Y**VATR*-208

Table 2

Lower limit of quantitation for phosphorylation level using the PPQ-MRM and direct-MRM methods

For each peptide the table indicates the lowest level of phosphorylation (in %) that can be accurately (80-120%) and reproducibly (CV <20%) detected by each method at the four different total peptide amounts (8, 40, 200 fmol or 1 pmol). The first number indicates the minimum level of phosphorylation that can be determined, with the actual amount detected shown in parentheses. Red indicates the lowest % of phosphorylation (other than 100%) determinable at the lowest total peptide level for each peptide.

Target Protein	Phosporylation site	Method	1 pmol	200 fmol	40 fmol	8 fmol
ERα (Estradiol receptor alpha, P03372	S154	direct-MRM	10 (8.5)	10 (9.1)	10 (8.8)	40 (33.6)
		PPQ-MRM	20 (20.0)	20 (21.8)	40 (46.8)	40 (37.8)
HER2 (Receptor tyrosine-protein kinase erbB-2, P04626)	Y1248	direct-MRM	100 (98.6)	100 (96.4)	40 (32.2)	40 (32.1)
		PPQ-MRM	20 (17.0)	20 (18.1)	100 (98.8)	100 (96.5)
HER2 (Receptor tyrosine-protein kinase erbB-2, P04626)	Y877	direct-MRM	40 (47.5)	40 (47.8)	100 (98.6)	100 (98.6)
		PPQ-MRM	10 (10.5)	10 (8.8)	20 (22.4)	20 (20.9)
RAF (proto-oncogene serine/threonine-protein kinase, P04049)	T491	direct-MRM	10 (9.1)	10 (10.4)	20 (20.2)	40 (45.5)
		PPQ-MRM	10 (9.3)	40 (43.6)	40 (47.7)	20 (17.9)
ERK1 (mitogen-activated protein kinase 3, P27361)	T202, Y204	direct-MRM	100 (99.2)	40 (47.2)	100 (98.5)	100 (96.6)
		PPQ-MRM	100 (99.4)	100 (98.9)	100 (99.5)	100 (98.3)

Table 3

Results of the analyses of ERα protein containing samples using the PPQ-MRM and direct-MRM methods

Two samples, one of an immunoprecipitated ERα protein from an un-induced MCF-7 breast cancer cell line, and one of an ERα protein expressed in an insect cell line were analyzed in duplicate using the PPQ-MRM and direct-MRM methods for the S154 phosphorylation site-containing peptide. Calculated amounts were determined using the highest MRM transition (doubly positive charged y_{11}) and were based on the known amount of SIS peptide added.

Source of ERα	Sample	Peptide detected	Calculated amount on column (fmol)	% phosphorylation
ERα from IP of MCF-7 cells	direct-MRM	EAGPPAFYRPN$_p$SDNR	0.00	0
		EAGPPAFYRPNSDNR	4.91	
	PPQ-MRM Untreated	EAGPPAFYRPNSDNR	5.79	−1.4
	PPQ-MRM Phosphatase	EAGPPAFYRPNSDNR	5.71	
Insect cell expressed ERα	direct-MRM	EAGPPAFYRPNpSDNR	112	10.2
		EAGPPAFYRPNSDNR	989	
	PPQ-MRM Untreated	EAGPPAFYRPNSDNR	953	8.6
	PPQ-MRM Phosphatase	EAGPPAFYRPNSDNR	1042	