Chapter 17

Measurement of Phosphorylated Peptides with Absolute Quantification

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Abstract

Mass spectrometry, when coupled to on-line separation such as liquid chromatography or capillary electrophoresis, enables the identification and quantification of protein expression and post-translational modification changes under diverse conditions. To date most of the methods for mass spectrometry-based quantification have either provided relative quantification information (e.g., comparison to a selected condition) or utilized one-point calibration curves, or calibration curves in a different biological matrix. Although these quantitative methods have been used to generate insight into the differences between biological samples, additional biological insight could be gained by accurately measuring the absolute quantity of selected proteins and protein modifications. To address this challenge, we have developed the MARQUIS (Multiplex Absolute Regressed Quantification with Internal Standards) method, designed to provide absolute quantification for potentially hundreds of peptides across multiple samples in a single analysis, using a multi-point internal calibration curve derived from synthetic, isotopically distinct standard peptides.

Key words Absolute quantification, Phosphorylation, EGFR, IMAC, LC-MS/MS

1 Introduction

Characterizing active signaling pathways in disease states has led to the development of many therapeutics that have significantly impacted clinical outcomes [1]. Central to these studies is the ability to identify and quantify levels of protein expression and post-translational modification (PTM). The myriad proteomics technologies designed for this task can be divided into two categories: recognition (usually by antibodies or aptamers) and physical measurement. Immunoblotting has been the most common proteomics approach for decades, and has recently been scaled up to facilitate high-throughput measurement in the form of protein microarrays [2]. However, recognition-based techniques are fundamentally constrained by their requirement of a priori knowledge, which prohibits identification of new targets. This is especially limiting in the context of PTMs, which are not hard coded into the

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genome and must first be identified experimentally. Mass spectrometry measures mass to charge ratios of thousands of peptides in complex mixtures, which can be used to identify novel sequences and PTMs. Recent quantification advances, including chemical-stable isotope labeling, metabolic-stable isotope labeling, and label-free quantification, have permitted comparison of thousands of proteins between multiple samples in a single analysis [3, 4]. Combining these methods with immunoaffinity enrichment against specific phosphorylation modifications has yielded extensive coverage of the phosphoproteome [5].

Despite these technical advances facilitating relative quantification, a key deficiency lies in the inability to measure the absolute amount of peptide, protein, or protein PTM in a cell. Stoichiometric information enables additional comparisons often yielding greater biological insight. For example, two proteins that show a twofold relative change could be increasing from 100 to 200 copies or 1 to 2 million copies; two scenarios which may have very different functional consequences. Additionally, absolute quantification of multiple phosphorylation sites indicates which are more prevalent, suggesting more probable interactions mediated by proteins' docking domains.

Previously, these measurements were difficult to obtain. An accurate quantification of protein expression with antibodies requires the use of recombinant standard proteins at known concentrations. Quantification of protein PTMs with this strategy is much more challenging due to the difficulty of establishing the modification state of the recombinant protein. Mass spectrometry offers a simple solution with the inclusion of synthetic standard peptides. One of the most common methods for obtaining absolute quantification is isotope dilution (commercially available as AQUA), in which a known amount of heavy-labeled synthetic peptide is added during processing, and quantification is obtained by comparing the elution profiles of the corresponding endogenous and standard peptides [6]. However, the reliance of a single point for calibration compresses the dynamic range of measurement and amplifies stochastic measurement errors. To address these issues we have developed an alternative method, termed Multiplex Absolute Regressed Quantification of Internal Standards (MARQUIS) [7]. This technique uses isotopically distinct synthetic standard peptides in conjunction with isobaric chemical labeling to create a multi-point internal calibration curve for each target peptide, including peptides with PTMs. Doing so, it faithfully compensates for nonlinear response due to multiple factors, including dynamic range of the instrument, signal-to-noise at the detection limits, and isobaric tag ratio compression that occurs from contaminants during precursor isolation.

The protocol presented here applies MARQUIS to quantify phosphorylation dynamics of the epidermal growth factor receptor (EGFR) signaling network in response to growth factor stimulation. This complex test case illustrates absolute quantification of key signaling targets rapidly changing within a cell. EGFR is a receptor tyrosine kinase capable of transmitting information from the extracellular environment to intracellular decision-making machinery that ultimately elicits behavioral responses. Ligand binding to the extracellular domain of the receptor induces dimerization and activation of the cytoplasmic kinase domain initiating several phosphorylation cascades that affect broad transcriptional programs. The resulting phenotypic changes include proliferation, migration, and differentiation [8]. Though previous work has catalogued the relative phosphorylation dynamics in response to a variety of growth factors, without stoichiometric information these studies have been limited in their ability to ascribe the individual contributions of particular signaling pathways to phenotypic response [9, 10]. Absolute quantification of phosphorylation dynamics in the EGFR signaling network has generated novel insight and yielded testable hypothesis about the structure of this signaling network [7].

The methods presented here are broadly applicable to a variety of systems, including other PTMs such as acetylation, methylation, and ubiquitination, or protein expression. We present standard peptide synthesis guidelines that should be considered during experimental design and highlight processes that can be adapted to multiple different mass spectrometry pipelines. These techniques will provide a new dimension to the application of mass spectrometry to proteomic studies.

2 Materials

2.1 Cell Culture (*See* Note 1)

1. MCF10A Human Mammary Epithelial Cells.
2. Complete Media: DMEM/F12 supplemented with 5 % Horse Serum, 20 ng/mL EGF, 0.5 μg/mL hydrocortisone, 100 ng/mL Cholera Toxin, 10 μg/mL Insulin, 1× Pen-Strep.
3. Starve Media: DMEM/F12 supplemented with 0.5 μg/mL hydrocortisone, 100 ng/mL Cholera Toxin, 1× Pen-Strep.
4. 1× Dulbecco's Phosphate-Buffered Saline (PBS).

2.2 Cell Stimulation and Lysis

1. 100 μg/mL EGF dissolved in MilliQ water.
2. Ice-cold PBS.
3. Urea Lysis Buffer: 8 M urea dissolved in MilliQ water, made immediately before use, kept on ice (*see* **Note 2**).
4. Cell scrapers.

2.3 Protein Reduction, Alkylation, Digestion

1. BCA Protein Assay.
2. Urea Lysis Buffer.
3. Synthetic standard peptides.
4. Ammonium Acetate Solution: 100 mM ammonium acetate (pH 8.9).
5. Dithiothreitol Solution: 1 M dithiothreitol in ammonium acetate solution.
6. Iodoacetamide Solution: 800 mM iodoacetamide in ammonium acetate solution.
7. Sequencing grade trypsin.

2.4 Peptide Desalting and Lyophilization

1. C-18 Sep-Pak cartridges.
2. Cleanup Equilibration Solution: 90 % acetonitrile in 0.1 % acetic acid.
3. Cleanup Elution Solution: 40 % acetonitrile in 0.1 % acetic acid.
4. Vacuum centrifuge.

2.5 TMT Labeling

1. Dried 800 μg TMT aliquots.
2. Dissolution Buffer: 0.5 M TEAB (triethylammonium bicarbonate, $N(Et)_3HCO_3$).
3. Anhydrous acetonitrile.
4. Ethanol (200 proof).
5. TMT Wash Solution: 40 % acetonitrile in 0.1 % acetic acid.
6. Vacuum centrifuge.

2.6 Precolumn Preparation

1. Fused silica capillary tubing OD 360 μm, ID 100 μm.
2. Silicon cutter.
3. Kasil.
4. YMC gel, ODS-A, 12 nm, S-10 μm, AA12S11 (10 μm beads).
5. Trypsin-digested angiotensin.

2.7 Immunoprecipitation

1. Protein G Plus agarose beads.
2. 4G10 anti-phosphotyrosine antibody, PT-66 anti-phosphotyrosine antibody, pY-100 anti-phosphotyrosine antibody.
3. TMT IP Buffer: 100 mM Tris–HCl, 1 % NP-40 (pH 7.4).
4. IP Buffer: 100 mM Tris–HCl, 0.3 % NP-40 (pH 7.4).
5. Tris Buffer: 500 mM Tris (pH 8.5).
6. IP Rinse Buffer: 100 mM Tris–HCl (pH 7.4).
7. IP Elution Buffer: 100 mM glycine (pH 2).

2.8 IMAC Enrichment

1. IMAC column: see Ref. 11 for details on preparing and testing IMAC columns.
2. EDTA: 100 mM EDTA (pH 8.9).
3. Iron Chloride: 100 mM iron (III) chloride.
4. Organic Rinse: 25 % acetonitrile, 1 % acetic acid, 100 mM NaCl.
5. IMAC Elution Buffer: 250 mM NaH_2PO_4 (pH 8.9).
6. HPLC Solvent A: 0.2 M acetic acid in ultrapure water.

2.9 LC-MS/MS Analysis

1. HPLC Solvent A.
2. HPLC Solvent B: 70 % acetonitrile, 0.2 M acetic acid in ultrapure water.
3. Thermo Scientific Easy-nLC 1000 in conjunction with a Thermo Scientific Q Exactive Orbitrap mass spectrometer.
4. MASCOT Distiller version 2.5 in conjunction with MASCOT Server version 2.4 (Matrix Science, Boston, MA).

2.10 Synthetic Peptide Design and Testing

1. Endogenous target peptide sequences.
2. Thermo Scientific Easy-nLC 1000 in conjunction with a Thermo Scientific Q Exactive Orbitrap mass spectrometer.
3. HPLC Solvent A.
4. HPLC Solvent B.

3 Methods

3.1 Cell Culture

1. Grow MCF10A cells in 10 cm dishes in 10 mL Complete Media at 37 °C and 5 % CO_2, splitting 1:4 when confluent.
2. Seed one plate per experimental condition in Complete Media for 48 h.
3. Aspirate Complete Media, rinse with 10 mL PBS, add 10 mL Starve Media.
4. Incubate cells at 37 °C for 24 h prior to stimulation.

3.2 Cell Stimulation and Lysis

1. Add growth factor directly to starve media to desired concentration, incubate at 37 °C for desired stimulation time.
2. With plate on ice, aspirate media, rinse cells with 10 mL ice-cold PBS to remove residual media, aspirate PBS.
3. Lyse cells by covering the dish with 1 mL Urea Lysis Buffer, using a cell scraper to remove adherent cells or remaining cell debris. Collect lysate in conical tube.
4. Vortex lysate, remove 10 μL aliquot for protein assay.
5. Store at −80 °C until further processing.

3.3 Protein Reduction, Alkylation, Digestion

1. Perform BCA Protein Assay with 10 µL lysate aliquot to determine protein concentration of each lysate (*see* **Note 3**).
2. Thaw lysate, add 400 µg protein from each sample into a fresh conical tube, and equalize sample volumes by adding Urea Lysis Buffer.
3. Add desired range of heavy-labeled synthetic peptides to samples (*see* **Note 4**).
4. Add 1:100 Dithiothreitol Solution to sample, to a final concentration of 10 mM dithiothreitol. Incubate at 56 °C for 1 h.
5. Add 1:14.5 Iodoacetamide Solution to sample, to a final concentration of 55 mM iodoacetamide. Incubate on rotor at room temperature for 1 h. Exposure of iodoacetamide to light should be limited by wrapping tubes in aluminum foil.
6. Dilute samples by adding 2.5× original lysate volume of ammonium acetate (e.g., 4 mL lysate would receive 10 mL ammonium acetate).
7. Add sequencing grade trypsin at a ratio of 1:50 (8 µg trypsin:400 µg lysate), allow digestion to proceed on rotor overnight (16 h) at room temperature.
8. Stop digestion by adding glacial acetic acid to 10 %.
9. Add any synthetic peptides containing missed cleavage sites at desired range.
10. Spin down samples to remove any debris that may clog Sep-Pak cartridge. Digested samples may be stored at −80 °C.

3.4 Peptide Desalting and Lyophilization

1. Acidify C-18 Sep-Pak cartridge (Waters WAT023501) with 10 mL 0.1 % acetic acid at a flow rate of 2 mL/min. Use a syringe pump for multiple samples, if available.
2. Equilibrate the cartridge with 10 mL Cleanup Equilibration Solution at a flow rate of 2 mL/min.
3. Wash the cartridge with 10 mL 0.1 % acetic acid at a flow rate of 2 mL/min.
4. Load the acidified peptide samples at a flow rate of 1 mL/min.
5. Wash the sample loaded cartridge with 10 mL 0.1 % acetic acid at a flow rate of 2 mL/min.
6. Elute the peptides into a clean conical tube with 10 mL Cleanup Elution Solution at 1 mL/min.
7. Reduce the total volume of each sample to less than 1 mL in a vacuum centrifuge.
8. Freeze the sample by immersing in liquid nitrogen for 10 min.
9. Lyophilize the sample overnight, or until all solvent has sublimated. Lyophilized peptides may be stored at −80 °C for several months.

3.5 TMT Labeling

1. Make peptide resuspension solution of 70 % ethanol and 30 % Dissolution Buffer.
2. Resuspend lyophilized peptides in 100 μL 70 % ethanol/30 % Dissolution Buffer, vortex for 1 min, and centrifuge at 12,000 × *g* for 1 min.
3. Add 40 μL anhydrous acetonitrile to each TMT aliquot, vortex for 1 min, centrifuge at 12,000 × *g* for 1 min.
4. Add resuspended TMT label to corresponding peptide sample. Vortex each sample for 1 min and centrifuge at 12,000 × *g* for 1 min.
5. Incubate for 1 h at room temperature.
6. Reduce the total volume of each sample to ~30 μL in vacuum centrifuge (approximately 30 min).
7. Aliquot 1 mL of 40 % acetonitrile in 0.1 % acetic acid in a fresh tube.
8. Combine all TMT samples into a single tube (*see* **Note 5**).
9. Add 40 μL TMT Wash Solution to each tube, vortex 1 min, centrifuge at 12,000 × *g* for 1 min, add rinse to sample tube.
10. Repeat rinsing procedure (done two times total).
11. Bring sample to dryness in vacuum centrifuge (small, dark pellet should form at the bottom of the tube).
12. Dried sample can be stored at −80 °C for several months.

3.6 Precolumn Preparation (*See* Note 6)

1. Cut approximately 20 cm long fused silica capillary with fused silica cutter.
2. Make frit mix by mixing Kasil and formamide (5:1) in micro-centrifuge tube, vortexing briefly and centrifuging at 12,000 × *g* for 1 min (*see* **Note 7**).
3. Dip one end of the column into the tube until material rises into the capillary about 0.5–1 cm.
4. Bake fritted columns at 100 °C for 10 min, ensuring that fritted end is not in contact with any surfaces.
5. Using a helium pressure injection cell (a.k.a. column packing bomb) on top of a magnetic stir plate, flush the column with acetonitrile at 400 psi for 5 min (*see* **Note 8**).
6. Resuspend small amount of YMC ODS-A beads in a glass vial containing 80 % acetonitrile/20 % isopropanol and add a magnetic stir bar.
7. Place the vial into the helium pressure injection cell with the stir plate turned on.
8. Pack beads with 500 psi until column bed length reaches 10 cm from the end of the frit.

9. Wash the column with 0.1 % acetic acid at 400 psi for 10 min.
10. Dry the column with helium at 400 psi for 10 min.
11. Cut dried column 1–2 cm from end of bead bed.
12. Prepare fresh frit mix.
13. Dip the second end of the column into the frit mix until material rises into the capillary about 0.5–1 cm.
14. Cure the second frit using a heat gun.
15. Wash the column with 0.1 % acetic acid at 400 psi for 10 min.
16. Condition precolumn with 500 fmol angiotensin.
17. Remove excess angiotensin by washing with acetonitrile at 400 psi for 5 min.
18. Remove organic solvent and recondition column by washing with 0.1 % acetic acid at 400 psi for 5 min.

3.7 Immunoprecipitation

1. Wash 60 µL Protein G Plus agarose beads with 300 µL IP Buffer. For all wash steps: combine in a microcentrifuge tube, place on rotator at 4 °C for 5 min, centrifuge at 4 °C for 1 min at 4000 × *g*, remove supernatant removing as much liquid but as few beads as possible using a gel loading pipette tip.
2. Resuspend beads with 300 µL IP Buffer and add 12 µg of each antibody to the washed beads.
3. Allow the mixture to incubate on a rotor at 4 °C for 6–8 h.
4. Wash the beads with 400 µL IP Buffer.
5. Resuspend TMT pellet in 400 µL TMT IP Buffer by vortexing.
6. Check pH of sample with 2 µL on pH strip, comparing with IP Buffer. If pH is lower than 7.4, add 5 µL of Tris Buffer, vortex, and measure again. Repeat until sample pH matches IP Buffer pH.
7. Add TMT sample to washed beads and incubate on rotor at 4 °C overnight (>12 h).
8. Centrifuge sample for 1 min at 4° C at 4000 × *g*, collect supernatant in a fresh microcentrifuge tube, store at −80 °C.
9. Wash the beads once with 400 µL TMT IP Buffer.
10. Wash the beads three times with 400 µL IP Rinse Buffer.
11. After final wash, add 70 µL of IP Elution Buffer and incubate at room temperature on rotor for 30 min.
12. Load eluted sample onto an IMAC column.

3.8 IMAC Enrichment

1. Prepare an IMAC column for metal affinity enrichment of phosphopeptides.
2. Rinse the IMAC column with EDTA Solution for 10 min at a flow rate of 10 µL/min.

3. Wash the IMAC column with MilliQ water for 10 min at a flow rate of 10 μL/min.
4. Load the IMAC column with Iron Chloride at a flow rate of 10 μL/min for 10 min.
   (a) Optional: Flip the column to flow in opposite direction at 10 min.
5. Rinse the IMAC column with 0.1 % acetic acid for 10 min at a flow rate of 10 μL/min.
6. To collect the non-retained, nonphosphorylated peptides, attach a flow-through precolumn to the IMAC with a Teflon connector, test junction by flowing 0.1 % acetic acid at 800 psi.
7. Determine pressure needed to generate a flow rate of 1 μL/min through the IMAC and precolumn in series.
8. Replace 0.1 % acetic acid with eluate from immunoprecipitation, load sample at 1 μL/min (*see* **Note 9**).
9. Remove precolumn containing flow-through peptides.
10. Rinse the IMAC column with Organic Rinse for 5 min at 10 μL/min (*see* **Note 10**).
11. Rinse with 0.1 % acetic acid for 5 min at 10 μL/min.
12. Place a fresh precolumn on the IMAC column with a Teflon connector, test junction by flowing 0.1 % acetic acid at 800 psi.
13. Determine pressure needed to generate a flow rate of 2 μL/min through the IMAC and precolumn in series.
14. Replace 0.1 % acetic acid with IMAC Elution Buffer, flow 40 μL Elution Buffer over IMAC and precolumn in series (*see* **Note 11**).
15. Rinse precolumn with HPLC Solvent A for 10 min prior to LC-MS/MS analysis.

3.9 LC-MS/MS Analysis

1. Analyze peptides eluted from IMAC by LC-MS/MS using reverse-phase chromatography performed in line with a Q Exactive mass spectrometer.
2. Elute peptides using a 120-min gradient (0–100 % HPLC Solvent A to Solvent B).
3. Acquire data using the mass spectrometer in targeted acquisition mode.
   (a) Acquire SIM scans at 70k resolution for each pair of endogenous peptide and its heavy isotope standard, with isolation window set to include both ions.
   (b) Acquire MS/MS scans for both endogenous peptide and heavy isotope standard. Typical settings include an MS1 isolation width of 2 m/z, MS2 fragmentation collision energy of 35.0, MS2 maximum ion injection time of 2 s,

and an AGC target of 3e6 (this large AGC target is chosen to maximize dynamic range).

4. Fragmentation of the synthetic peptide produces a standard curve, with calibration points covering the concentration range of peptides that were originally added to each biological sample. This step also provides a control for the linear dynamic range and noise floor of the TMT marker ions.
5. Total endogenous peptide is calculated by comparing the signal intensity of endogenous peptide with the standard peptide in the SIM scan.
6. Endogenous peptide concentrations in each sample can be calculated by apportioning the total amount of endogenous peptide between input conditions using the fractional reporter ion intensities generated by MS2 fragmentation of the endogenous peptide precursor.

3.10 Synthetic Peptide Design and Testing

1. Peptides should be synthesized containing identical sequences to endogenous target peptides, according to the specificity of the selected proteolytic enzyme: e.g., for trypsin, peptides would span from the residue immediately prior to the N-terminal K or R residue to the C-terminal K or R residue.
2. Peptides must contain at least one (but can have many) heavy isotope encoded amino acid residues. Note that larger peptides might require two heavy isotope encoded amino acid residues to ensure adequate separation between the endogenous and synthetic peptides during precursor isolation.
3. Synthetic peptides should be quantified by amino acid analysis to obtain accurate concentrations.
4. Multiple standard peptides may be pooled to create a single peptide cocktail that can be added to lysates.
5. Peptides containing frequently occurring missed cleavage sites may also be synthesized (these may also be pooled to create a second mixture, but should be kept separate from standard tryptic peptide cocktail to be added after the digestion step).
6. Analyze standard peptide mixture using reverse-phase chromatography performed in line with a Q Exactive mass spectrometer, eluting peptides with a 120-min gradient (0–100 % HPLC Solvent A to Solvent B).
7. Determine target peptide elution windows from extracted ion chromatogram (XIC) using calculated peptide precursor m/z ratios.
8. Create Inclusion List for targeted MS analysis. This should include:

(a) Peptide precursor m/z ratios for synthetic standard peptides and endogenous peptides at multiple potential charge states (e.g., +2, +3)

(b) Elution start and end times, as determined from **step 7**.

4 Notes

1. Cell culture can be performed with a variety of cell lines, with a minimum necessary protein content of 400 μg per sample.
2. (Optional) Add 1 mM activated sodium orthovanadate to prevent phosphatase activity.
3. (Optional) In addition to protein standard, sample lysates may be compared to control lysates of known cell counts to obtain measurement of cells/μg. This can be later used to convert peptide measurement to copies/cell.
4. Example TMT 10plex scheme: 3 pmol, 1 pmol, 300 fmol, 100 fmol, 30 fmol, 10 fmol, 3 fmol, 1 fmol, 0.3 fmol, 0.1 fmol.
5. Use one pipette tip for all combining and washing steps to minimize sample loss.
6. IMAC enrichment is also compatible with commercial precolumn setups.
7. Frit mix will polymerize with time, so this step should be performed quickly. Overly polymerized mixture will not rise into the column.
8. For columns that do not flow immediately, use silicon cutter to cut a small piece of the fritted end of the column.
9. Eluate need not be removed from microcentrifuge tube with beads. Frits prevent agarose beads from flowing through columns.
10. (Optional) Flip the column to flow in opposite direction at 5 min to decrease nonspecific binding.
11. For autosampler setups, sample may be collected in a fresh autosampler vial placed inverted on top of the IMAC column.

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