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Quantitative Proteomic Analysis of Phosphotyrosine-Mediated Cellular Signaling Networks

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Summary

Receptor tyrosine kinases receive extracellular cues, such as ligand binding, and transmit this information to the cell through both autophosphorylation and phosphorylation of tyrosine residues on selected substrates, stimulating a variety of signal transduction pathways. Quantitative features, including intensity, timing, and duration of phosphorylation of particular residues, may play a role in determining cellular response, but experimental data required for analysis of these features have not previously been available. We have recently developed a methodology enabling the simultaneous quantification of tyrosine phosphorylation of specific residues on dozens of key proteins in a time-resolved manner, downstream of receptor tyrosine kinase activation. In this chapter, we present a detailed description of this mass spectrometry-based method, including conditions for cell culture and stimulation, sample preparation for stable isotope labeling and peptide immunoprecipitation, immobilized metal affinity chromatography–liquid chromatography–tandem mass spectrometry analysis of affinity-enriched tyrosine phosphorylated peptides, and analysis of the resulting MS data.

Key Words: Signal transduction; tyrosine phosphorylation; immobilized metal affinity chromatography; liquid chromatography; mass spectrometry.

1. Introduction

Protein phosphorylation-mediated cellular signaling networks regulate information flow within the cell through activation and inhibition of kinases and phosphatases leading to dynamic, reversible protein posttranslational modification. Information contained within these signaling networks, and therefore biological response to an initial stimulus, can vary depending on three key

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factors: intensity, timing, and duration of the signal. Quantifying these factors has been limited by another problem, in that it has been challenging to determine the sites of protein posttranslational modification that control how signals are propagating through the system. However, significant advances over the past several years have been made in the field of phosphoproteomics, such that it is now possible to catalog hundreds to thousands of protein phosphorylation sites from a given sample *(1,2)*. These advances have now made it possible to focus on analyzing the temporal dynamics of protein phosphorylation events following a perturbation to the system. In one example, stable isotope labeling in cell culture (SILAC) *(3)* has been combined with phosphopeptide enrichment through two rounds of affinity chromatography. This approach was used to quantify intensity changes for hundreds of phosphorylation sites on yeast proteins following α-factor stimulation *(4)*. Although this study only looked at a single time point following stimulation, others have used SILAC to quantify changes in total protein tyrosine phosphorylation at selected time points after epidermal growth factor (EGF) stimulation *(5)*.

In this chapter, we describe a method by which protein tyrosine phosphorylation levels may be quantified on multiple samples in a single analysis with site-specific resolution. In the method, biological samples are lysed by a protein denaturant, proteins are enzymatically digested to peptides and labeled with an amine-specific stable isotope-coded reagent (iTRAQ) *(6)*. Following labeling and mixing of the samples, tyrosine phosphorylated peptides are immunoprecipitated with a pan-specific anti-phosphotyrosine antibody *(7)*. After elution from the antibody, phosphorylated peptides are further enriched through immobilized metal affinity chromatography (IMAC) *(8)*, passed to a reverse-phase column, and analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). We have applied this method to analyze the temporal dynamics of tyrosine phosphorylation following EGF stimulation, generating temporal phosphorylation profiles for 78 tyrosine phosphorylation sites on 58 proteins in a single IMAC-LC–MS/MS analysis *(9)*. Although the specifics of the method apply directly to the analysis of epidermal growth factor receptor (EGFR) signaling in human mammary epithelial cells, the general approach should be applicable to a broad variety of systems.

2. Materials

2.1. Cell Culture and Lysis

1. DFCI-1 medium: 1:1 (v/v) MEM-α: Ham-F12 (without sodium bicarbonate) supplemented with 2 m*M* L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 1% (v/v) fetal bovine serum, 1 µg/mL insulin, 10 µg/mL transferring holo form, 50 µ*M* of freshly made ascorbic acid (all from GIBCO, Carlsbad, CA), 1% (v/v) bovine pituitary extract (Pelfreez Biologicals, Rogers, AZ), 10 m*M* HEPES,

2.8 μ*M* hydrocortisone (dexamethasone), 2 n*M* β-estradiol, 15 n*M* sodium selenite, 0.1 m*M* ethanolamine, 0.1 m*M* *o*-phosphoethanolamine, 10 n*M* triodothyronine, 1 ng/mL cholera toxin (all from Sigma, St. Louis, MO), 19 m*M* sodium bicarbonate (Mallinckrodt, Hazelwood, MO), 12.5 ng/mL EGFR (Peprotech, Rocky Hill, NJ). The pH should be 7.4 in a 5% CO_2 incubator ***(10)***.

2. Serum-free DFCI-1, with the same components as DFCI-1 medium (**step 1**) minus fetal bovine serum, bovine pituitary extract, and EGF. Supplement with 1 mg/mL of bovine serum albumin (Sigma).
3. Phosphate-buffered saline (PBS) (Gibco).
4. 10X trypsin-EDTA solution (Sigma), diluted to 1X in PBS.
5. Lysis buffer consisting of 8 *M* urea and 1 m*M* activated sodium orthovanadate (Sigma). After preparation, the solution should be buffered to pH 7.0 at room temperature.
6. Cell lifters (Corning, Corring, NY).
7. Micro BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL).

2.2. Protein Digestion and Sample Preparation

1. Dithiothreitol (Sigma).
2. Iodoacetamide (IAc) (Sigma) (light sensitive).
3. Sequencing grade-modified trypsin (Promega, Fitchburg, WI).
4. Trypsin digestion buffer: 100 m*M* ammonium acetate (Sigma), pH 8.9.
5. C18 Sep-Pak Plus cartridges (Waters, Milford, MA).
6. Glacial acetic acid (Mallinckrodt).
7. 0.1% acetic acid solution (Mallinckrodt).
8. 0.1% acetic acid and 25% acetonitrile (ACN) solution (Mallinckrodt).
9. 0.1% acetic acid and 40% ACN solution (Mallinckrodt).
10. 0.1% acetic acid and 90% ACN solution (Mallinckrodt).

2.3. iTRAQ Stable Isotope Labeling and Phosphotyrosine Peptide Immunoprecipitation

1. 0.5 *M* triethylammonium biocarbonate ($N[Et]_3HCO_3$), pH 8.5 (Sigma).
2. Ethanol (Mallinckrodt).
3. iTRAQ reagent multiplex kit (Applied Biosystems, Foster City, CA).
4. IP buffer: 100 m*M* Tris-HC1 100 m*M* NaCl (both from Sigma), and 0.3% NP40, pH 7.4 (Fluka, Buchs SG, Switzerland).
5. 0.5 *M* Tris-HC1 buffer, pH 8.5.
6. Rinse buffer: 100 m*M* Tris-HC1 and 100 m*M* NaCl, pH 7.4.
7. Elution buffer: 100 m*M* glycine (Sigma), pH 2.5.
8. PY99 Immobilized anti-phosphotyrosine antibody, store at 4°C (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

2.4. IMAC and LC–MS/MS

1. IMAC packing material: POROS 20 MC (Applied Biosystems).
2. 100 m*M* EDTA (Sigma), pH 8.5.

3. 100 m*M* $FeCl_3$ (Sigma).
4. 0.1% acetic acid solution (Mallinckrodt).
5. IMAC organic rinse solution: 25% MeCN, 1% acetic acid, and 100 m*M* NaCl.
6. IMAC elution buffer: 250 m*M* Na_2HPO_4, pH 8.0.
7. High-performance liquid chromatography solvent A: H_2O/acetic acid, 99/1 (v/v).
8. High-performance liquid chromatography solvent B: H_2O/MeCN/acetic acid, 29/70/1 (v/v).
9. Fused silica capillary (360 μm outer diameter [O.D.] × 50 μm inner diameter [I.D.]), (360 μm O.D. × 100 μm I.D.), and (360 μm O.D. × 200 μm I.D.) (Polymicro Technologies, Phoenix, AZ).
10. YMC ODS-A 10 μm packing material (Kanematsu Corp., Tokyo, Japan).
11. YMC ODS-AQ 5 μm packing material (Waters).

3. Methods

3.1. Cell Culture and EGF Stimulation

1. Passage 184A1 human mammary epithelial cells at 80–90% confluence with 1X trypsin-EDTA in order to provide new maintenance cultures on 100-mm tissue cultures plates, and experimental cultures on 150-mm tissue culture plates. To generate an experimental plate approaching confluence after 24 h, incubate the cells obtained from 1.5 100-mm tissue cultures plates (~5×10^6 cells) into one 150-mm tissue culture plate.
2. After reaching confluence in the experimental plate, wash the cells with PBS and incubate with serum-free DFCI-1 for 12 h.
3. After 12 h serum starvation, the cultures are washed with PBS and incubated with 25 n*M* EGF diluted in serum-free DFCI-1 for the desired time. For instance, in **Fig. 1**, four plates of cells were incubated with EGF for 0, 5, 10, or 30 min.

3.2. Preparation of Samples for Phosphotyrosine Peptide Immunoprecipitation

1. Following cell culture and agonist (EGF) incubation, the cultures are placed on ice, washed with PBS, and lysed with 3 mL of lysis buffer.
2. Lysates are collected into 15-mL conical tubes and spun down at 1000*g* for 10 min.
3. A 10-μL aliquot from each sample is taken to perform protein concentration assay, using the bicinchoninic acid assay (micro BCA kit) according to the manufacturer's protocol.
4. The sample lysates are reduced by incubation for 1 h with dithiothreitol at 56°C. Reduction is followed by alkylation by incubation for 1 h in the dark (IAc is sensitive to light) at room temperature with 55 m*M* IAc.
5. Following alkylation, the samples are diluted four times by addition of 10 mL of trypsin digestion buffer, and digested overnight with 40 μg of trypsin (~1:100 trypsin:substrate ratio). Digestion is terminated by acidifying the solution to pH 3.0 with 500–1000 μL of glacial acetic acid.

6. Sep-Pak Plus cartridges are preconditioned by sequentially flowing at a rate of 2 mL/min:
   a. 10 mL of 0.1% acetic acid solution.
   b. 10 mL of 0.1% acetic acid and 90% ACN solution.
   c. 10 mL of 0.1% acetic acid solution.
7. Once the Sep-Pak Plus cartridges had been conditioned, the sample lysates are loaded into different cartridges at a rate of 1 mL/min.
8. Both the hydrophilic and hydrophobic fractions of peptides are recovered by sequential elution with 10 mL of 0.1% acetic acid, 25% ACN solution, and 10 mL of 0.1% acetic acid, 40% ACN solution.
9. The recovered fractions are aliquoted into 1-mL aliquots and concentrated to 100–200 µL volume using a vacuum centrifuge prior to overnight lyophilization. Dried aliquots are stored at –80°C until needed.

3.3. iTRAQ Stable Isotope Labeling and Phosphotyrosine Peptide Immunoprecipitation

1. One aliquot of each sample (control, 5, 10, and 30 min) is dissolved with 30 µL of 0.5 *M* triethylammonium bicarbonate ($N[Et]_3HCO_3$), pH 8.5.
2. Thaw a set of iTRAQ labeling reagents (stored at –80°C) to room temperature. Each of the four iTRAQ reagents is dissolved with 70 µL of ethanol, and transferred into the corresponding sample (for instance, in **Fig. 1**, 114 was added to the control sample, 115 was added to the 5-min sample, 116 was added to the 10-min sample, and 117 was added to the 30-min sample).
3. Mix each sample and centrifuge (2800*g* for 5 min). Reaction proceeds at room temperature for 1 h (*see* **Note 1**).
4. After 1 h, each sample is concentrated to approx 30 µL in a vacuum centrifuge.
5. The four samples are combined, reduced to complete dryness in a vacuum centrifuge, and stored at –80°C.
6. 200 µL IP buffer is used to rinse 10 µg of immobilized PY99 for 5 min at 4°C. Spin down the antibody beads at 2800*g*, and remove the supernatant.
7. Dissolve the iTRAQ-labeled sample in 150 µL of IP buffer and 300 µL of water.
8. Adjust the pH of the iTRAQ-labeled sample to pH 7.4 with 0.5 *M* Tris buffer, pH 8.5, and mix with rinsed antibody. Incubate the sample and antibody mixture overnight at 4°C while rotating (*see* **Note 2**).
9. The antibody beads are spun down at 6000 rpm for 5 min. Remove the supernatant and store at –80°C. The antibody beads are then rinsed three times with 400-µL rinse buffer at 4°C for 5 min. The peptides are eluted with 60 µL of 100 m*M* glycine, pH 2.5 at room temperature for 25 min while rotating. The antibody beads are spun down at 2800*g* for 5 min. The eluted peptides are transferred into a new microcentrifuge tube.

3.4. IMAC and LC–MS/MS

1. Clean and condition an IMAC column (a 10-cm long self-packed IMAC (20MC, Applied Biosystems) capillary column (200 µm I.D., 360 µm O.D. was used to generate the data shown in **Fig. 2**) by passing each of the following solutions

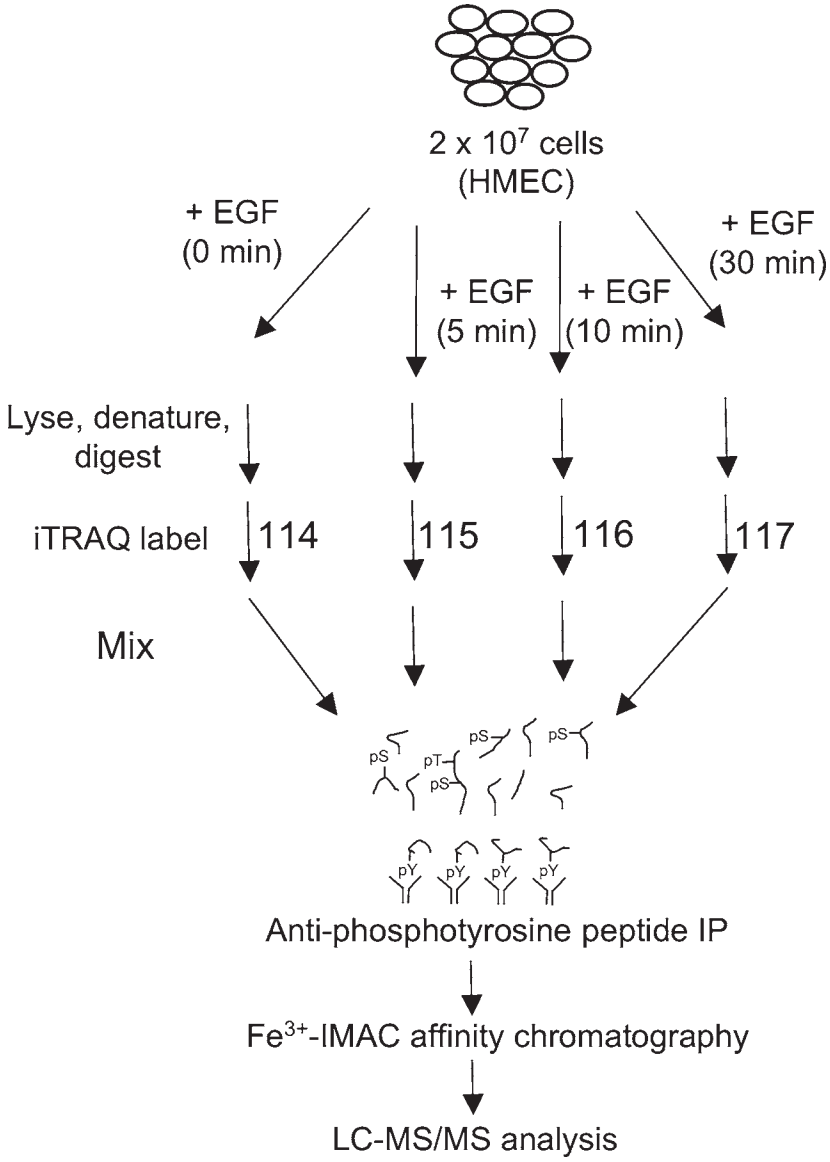

Fig. 1. Schematic representation of mass spectrometry-based approach to analysis of temporal phosphorylation on specific tyrosine residues. Four plates of human mammary epithelial cells were cultured under normal conditions, serum starved for 12 h, and stimulated with 25 n*M* epidermal growth factor for 0, 5, 10, or 30 min. Following cell lysis, proteins were enzymatically digested and the resulting peptide mixture was desalted prior to aliquoting into ten equivalent fractions per stimulation time. For each stimulation time, one aliquot was labeled with one of the four isoforms of the (iTRAQ

through the column at a flow rate of 5 µL/min for 10 min: 100 m*M* EDTA, H_2O, 100 m*M* $FeCl_3$, and 0.1% acetic acid.

2. Peptides eluted from antibody are loaded onto the conditioned IMAC capillary column at 1–2 µL/min.
3. To remove nonspecifically retained peptides, the IMAC column is rinsed with organic rinse solution for 10 min at a flow rate of 5 µL/min.
4. Equilibrate the IMAC column with 0.1% acetic acid for 10 min at a flow rate of 5 µL/min. Connect the IMAC column to a reverse-phase precolumn (we typically use capillary precolumn of dimension 100 µm I.D., 360 µm O.D., packed with 10-cm of YMC ODS-A [10 µm] [Kanematsu Corp.]).
5. Peptides are eluted from the IMAC column onto the reverse-phase capillary precolumn with 50-µL elution buffer. To remove excess phosphate buffer prior to MS analysis, rinse the precolumn with 0.1% acetic acid for 10 min at a flow rate of 1–2 µL/min. After rinsing, connect the precolumn to a reverse-phase analytical column (to generate the data shown in **Fig. 2**, a 10-cm long self-packed C18 [YMC-Waters 5 µm ODS-AQ] analytical capillary column [50 µm I.D., 360 µm O.D.] with an integrated electrospray tip [~1 µm orifice] was used) (*see* **Note 3**).
6. Peptides are eluted from the reverse-phase columns using the following gradient: 10 min from 0 to 15% B, 75 min from 15 to 40% B, and 15 min from 40 to 70% B. Column flow rate should be set to optimum flow rate for the given analytical column depending on column diameter and electrospray emitter tip diameter.
7. As they elute from the column, peptides are directly electrosprayed into a quadrupole time-of-flight mass spectrometer (QSTAR XL Pro, Applied Biosystems). The instrument is operated in information-dependent acquisition mode, in which a full scan mass spectrum is acquired followed by MS/MS spectra of the five most intense peaks with charge state of 2–5. The instrument is set to exclude previously selected peaks for 40 s.
8. To normalize for sample labeling and mixing, a small amount (0.5 µL) of the supernatant from the peptide IP is loaded onto a conditioned IMAC capillary column and analyzed similarly (i.e., repeat **steps 3–7**).

3.5. Data Analysis and Validation

1. MS/MS spectra are extracted (Mascot.dll) and searched against human protein database (NCBI) using ProQuant (Applied Biosystems). Prior to searching, an interrogator database is generated by predigesting the human protein database with trypsin and allowing one missed cleavage and up to six modifications on a single peptide (phosphotyrosine ≤2, phosphoserine ≤1, phosphothreonine ≤1,

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Fig. 1 *(continued from opposite page)* reagent and then mixed with the labeled aliquots from the other time points. Phosphotyrosine-containing peptides were immunoprecipitated and further enriched by immobilized metal affinity chromatography prior to liquid chromatography–tandem mass spectrometry analysis.

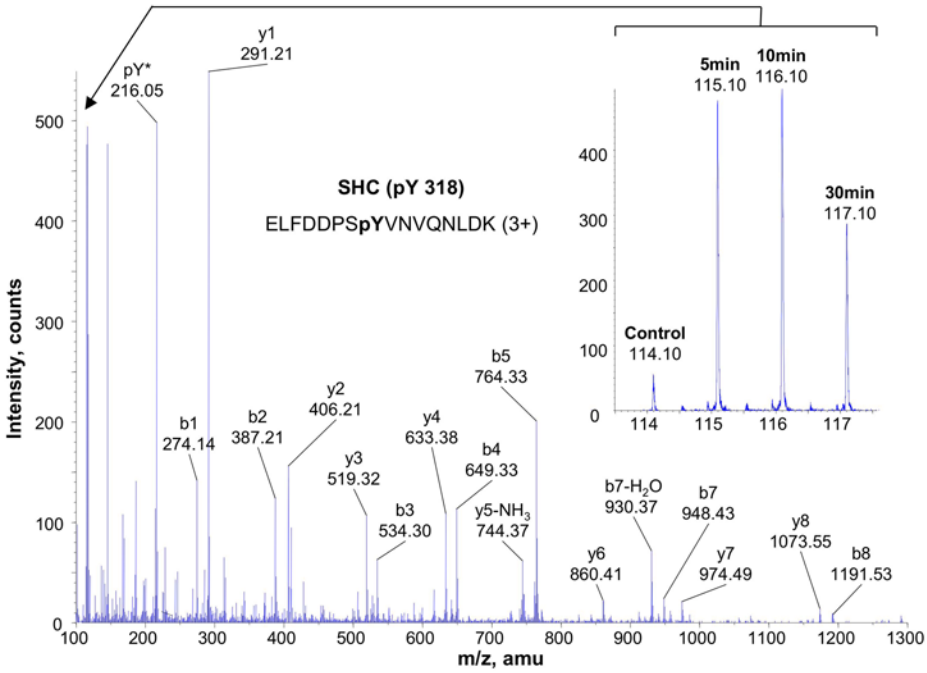

Fig. 2. A representative tandem mass spectrometry (MS/MS) spectrum for one of the tyrosine phosphorylated peptides identified in the analysis of epidermal growth factor-stimulated human mammary epithelial cells. Precursor ion of *m/z* ratio 755.4 with +3 charge state was selected for fragmentation from the full scan mass spectrum. From the resulting MS/MS spectrum, y- and b-type fragment ions enabled peptide identification and phosphorylation site assignment, while peak areas for each of the iTRAQ marker ions (inset, with mass labels and corresponding stimulation time points) enabled quantification of the temporal phosphorylation profiles.

iTRAQ-lysine ≤4, and iTRAQ-tyrosine ≤4). For the database search, mass tolerance is set to 0.15 amu for precursor ions and 0.1 amu for fragment ions.

2. Phosphotyrosine-containing peptides identified from the database search are manually validated by confirming the assignment of y, b, and a-type ions, as well as neutral loss (of H_2O, NH_3, or H_3PO_4 from serine- or threonine-phosphorylated peptides). Peptide sequences are accepted only when all major peaks in the MS/MS spectra can be assigned (*see* **Note 4**).
3. In order to quantify the amount of phosphorylation on a given peptide across the four samples, peak areas for each of the four iTRAQ signature peaks (*m/z*: 114.1, 115.1, 116.1, 117.1) are obtained from the MS/MS spectrum. To account for isotopic overlap, peak areas must be corrected according to the manufacturer's instructions.

4. To correct for sample labeling and mixing, MS/MS data from the IMAC-LC–MS/MS analysis of the supernatant is searched using the same parameters (*see* **step 1**). From the search results, 20 nonphosphorylated peptide hits from abundant proteins are selected and confirmed (*see* **step 2**).
5. In order to normalize the data, one of the iTRAQ marker ions must be selected as the standard, and all quantification is then made relative to the peak area of this ion (for instance, in **Fig. 2** the 5 min sample [the 115 peak] was used as the normalization standard, as this peak had the greatest signal-to-noise ratio and therefore the lowest noise-associated error). The average ratio from the twenty nonphosphorylated peptides reflects the variation in the starting amount of the four samples, and is used to further correct the values of phosphotyrosine-containing peptides.
6. Mean phosphorylation, standard deviation, and *p*-values to estimate statistical significance for differential phosphorylation between the different time points are calculated using Excel. The *p*-values are calculated using a paired, two-tailed student test.
7. To cluster phosphorylation sites with self-similar profiles, a self-organizing map is generated with the Spotfire program. Excel spreadsheets containing the quantification results from the MS data are loaded into Spotfire, the self-organizing map option is selected, and the architecture of the self-organizing map is selected from the options in the window.

4. Notes

1. The recommended peptide amount is 100 μg for each tube of iTRAQ labeling reagent. Incomplete labeling could occur if excess amount of peptides is used. The sample amount should be kept fairly constant for labeling with each of the four iTRAQ isoforms.
2. The quality of phosphotyrosine antibody is crucial for the success of the method. We have observed that many antibodies lose specificity over time even when stored at 4°C. A standard sample should be used to check the quality of the antibody immediately after receipt.
3. Selection of reverse-phase columns will dramatically affect performance, especially with regard to detection limits of the analysis, a crucial point for detecting low-level tyrosine phosphorylated peptides. We typically use custom-made columns in the format originally published by Martin et al. ***(11)***. Electrospray emitter tips are generated on these columns used a Sutter P-2000 laser puller, and typically range from 1 to 2 μ, providing optimal flow rates at 20–50 nL/min.
4. Sample complexity may result in incorrect quantification when using the iTRAQ stable isotope-labeling reagents. This problem manifests when multiple peaks are within the *m/z* isolation window of the selected precursor ion. Under these conditions, multiple peaks are isolated and fragmented simultaneously, with each contributing to the peak areas of the iTRAQ marker ions (*m/z* 114–117). In this case, the relative ratios of the four signature peaks are no longer representative of the relative ratios of the selected precursor ion in the four samples. To avoid such errors in quantification, sample complexity should be decreased through frac-

tionation and the corresponding MS and MS/MS spectrum should be examined for possible contaminants.

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References

1. Ficarro, S. B., McCleland, M. L., Stukenberg, P. T., et al. (2002) Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae. *Nat. Biotechnol.* **20,** 301–305.
2. Beausoleil, S. A., Jedrychowski, M., Schwartz, D., et al. (2004) Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc. Natl. Acad. Sci. USA* **101,** 12,130–12,135.
3. Ong, S. E., Blagoev, B., Kratchmarova, I., et al. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell Proteomics* **1,** 376–386.
4. Gruhler, A., Olsen, J. V., Mohammed, S., et al. (2005) Quantitative Phosphoproteomics Applied to the Yeast Pheromone Signaling Pathway. *Mol. Cell Proteomics* **4,** 310–327.
5. Blagoev, B., Ong, S. E., Kratchmarova, I., and Mann, M. (2004) Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. *Nat. Biotechnol.* **22,** 1139–1145.
6. Ross, P. L., Huang, Y. N., Marchese, J., et al. (2004) Multiplexed protein quantitation in saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Mol. Cell Proteomics* **3,** 1154–1169.
7. Rush, J., Moritz, A., Lee, K. A., et al. (2004) Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nat. Biotechnol.* **23,** 94–101.
8. Andersson, L. and Porath, J. (1986) Isolation of phosphoproteins by immobilized metal (Fe3+) affinity chromatography. *Anal. Biochem.* **154,** 250–254.
9. Zhang, Y., Wolf-Yadlin, A., Ross, P. L., et al. (2005) Time-Resolved Phosphotyrosine Analysis Reveals Dynamic Modules in EGFR Signaling. *Mol. Cell Proteomics* **4,** 1240–1250.
10. Band, V., Zajchowski, D., Kulesa, V., and Sager, R. (1990) Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. *Proc. Natl. Acad. Sci. USA* **87,** 463–467.
11. Martin, S. E., Shabanowitz, J., Hunt, D. F., and Marto, J. A. (2000) Subfemtomole MS and MS/MS peptide sequence analysis using nano-HPLC micro-ESI fourier transform ion cyclotron resonance mass spectrometry. *Anal. Chem.* **72,** 4266–4274.