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# Detection of Post-translational Modifications by Fluorescent Staining of Two-Dimensional Gels

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**Summary** Post-translational modifications (PTMs) are key to the regulation of functional activities of proteins. Quantitative and qualitative information about PTM stages of proteins is crucial in the discovery of biomarkers of disease. Recent commercial availability of fluorescent dyes specifically staining PTMs of proteins such as phosphorylation and glycosylation enables the specific detection of protein regulations taking place with respect to these modifications. Activity and molecular and signalling interactions of many proteins are determined by their extent of phosphorylation. In our search for biomarkers of neurodegenerative diseases such as Multiple Sclerosis (MS), using its animal model, Experimental autoimmune encephalomyelitis (EAE), we have applied the phosphorylation specific fluorescent dye, ProQ Diamond, to study changes taking place in the phosphoproteome. Subsequent Colloidal Coomassie staining of the same gels detects the changes at the whole proteome level. We have detected many changes taking place in the CNS tissue of the EAE animals at the whole proteome as well as at the phosphoproteome level that has given valuable insights into the pathophysiological mechanism of EAE and possibly also MS.

**Key Words** Phosphoproteome; ProQ Diamond; 2D gel electrophoresis; fluorescent stain; in-gel digestion; peptide extraction.

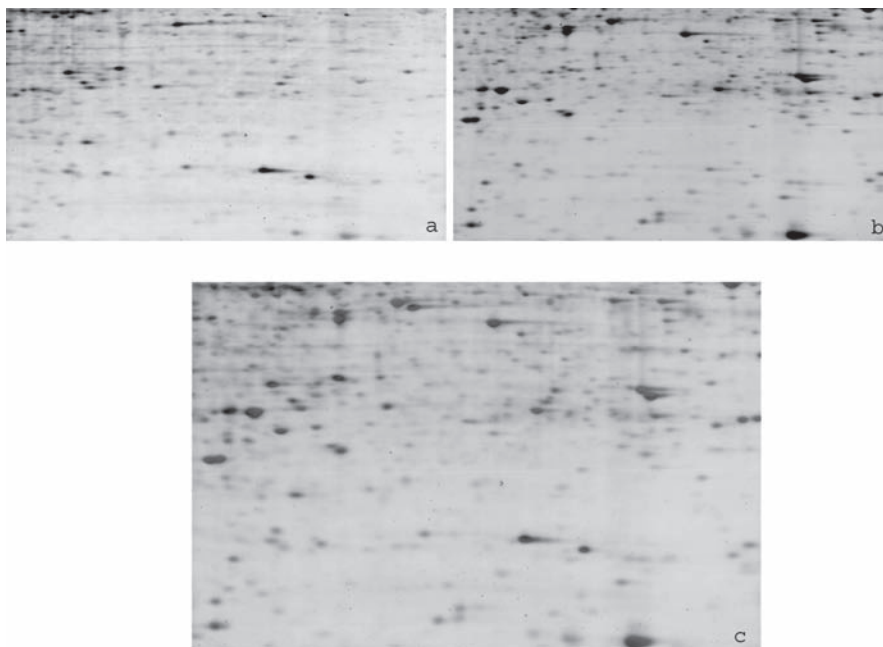
## 1 Introduction

The analysis of post-translational modifications is of high significance in proteomic studies aimed at the discovery of protein markers relevant in the pathogenesis of diseases. Although limited in the coverage of the whole proteome, one of the main strengths of 2-dimensional polyacrylamide gel electrophoresis (2D PAGE) is the ability to visualize protein isoforms. However, because of their very low stoichiometry, the post-translationally modified isoforms often remain undetected on 2D gels using classical staining methods. Commercially available fluorescent stains such as, ProQ Emerald, (Molecular Probes) and ProQ Diamond, (Molecular Probes)

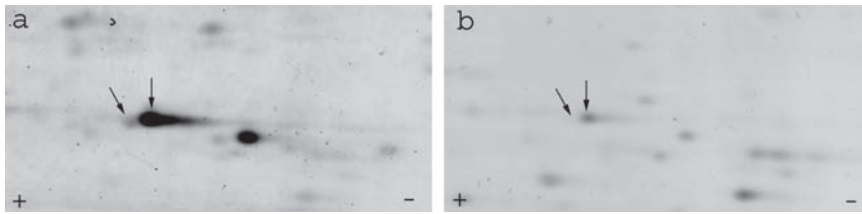
have facilitated the specific detection and identification of glycosylation and phosphorylation of proteins separated on 2D gels, respectively. Proteins separated on 2D gels for proteomic analysis can be stained first by ProQ and subsequently by Colloidal Coomassie stain or any other protein stain.

ProQ Diamond stain binds specifically to proteins with phosphate groups on serine, threonine, and tyrosine residues. Because of the fluorescent nature ProQ Diamond stain can detect phosphorylated proteins present in as low as 4 ng per spot. The staining intensity correlates with the number of phosphate groups present in the respective proteins (1,2). This high sensitivity is especially very critical in the case of phosphorylated proteins because of their very low abundance (3,4). ProQ Diamond staining allows the comparative expression profiling of the phosphoproteome both in a quantitative and qualitative manner. Moreover, its high sensitivity increases the proteome coverage. As the dye binds noncovalently to the phosphate groups, it is compatible with subsequent mass spectrometric analysis.

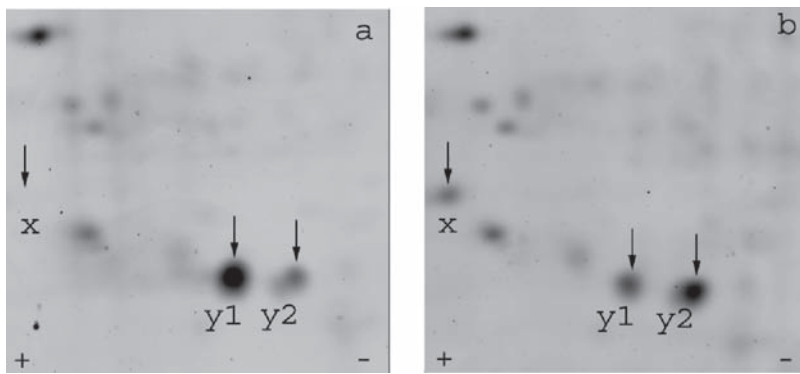
Mouse CNS tissue protein extracts are separated on 2D gels and stained for phosphoproteome and whole proteome (Figs. 2.1a and 2.1b). The two images are digitally colored and overlaid (Fig. 2.1c) to determine the relative position of the phosphoproteins on the whole protein stained image. The spots of interest are excised and prepared for mass spectrometry analysis. Thus, this method detects in parallel the expression level changes and altered phosphorylation modifications taking place under different physiological conditions (Figs. 2.2 and 2.3).



**Fig. 2.1** Phosphoproteome and whole proteome. (a) ProQ Diamond stained image of mouse spinal cord 2D gel. (b) Image of the same gel stained with Coomassie Blue. (c) The 2 images overlaid on top of each other. Image C is used to determine the relative position of the ProQ Diamond stained spots on the Coomassie stained image for spot picking



**Fig. 2.2** (a) ProQ Diamond stained gel image. (b). Same gel stained subsequently with Coomassie stain. The arrows indicate a protein that migrates at two different positions on a 2D gel. The more acidic spot is visible only on the ProQ Diamond stained image



**Fig. 2.3** Quantitative and qualitative differences of the phosphoproteome between control and EAE mouse brain. (a) 2D gel image of EAE brain proteins stained with ProQ. (b) 2D gel image of control brain proteins stained with ProQ. Spot **x** is up-regulated in EAE, an example for a quantitative difference in expression. Spots **y1** and **y2** represent the same protein. It differs between diseased and control animals in its extent of phosphorylation, a typical example of a phosphorylation change, where the protein moves more to the acidic end

## 2 Materials

### 2.1 Sample Preparation of Brain and Spinal Cord Sample for 2-Dimensional Electrophoresis

Unless otherwise mentioned all reagents are purchased from Bio-Rad, Hercules, CA

1. Isoelectric focussing (IEF) buffer: 7M urea, 2M thiourea, 100mM dithiothreitol (DTT), 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 0.05% biolytes 3–10, 0.001% (v/v) bromophenol blue (for color). Prepared and stored as 1-mL aliquots at  $-80^{\circ}\text{C}$ . Once thawed should not be frozen again (*see Note 1*).
2. Protease inhibitors: All protease inhibitors are added to the IEF buffer in 1  $\times$  concentration just before use.

- a. Pepstatin (Roche): 1000× stock prepared by dissolving 1 tablet in 1 mL ethanol and stored at  $-20^{\circ}\text{C}$  up to 3 mo.
  - b. Complete (Roche): 25× stock prepared by dissolving 1 tablet in 2 mL Double distilled water and stored in aliquots at  $-20^{\circ}\text{C}$  up to 3 mo.
  - c. Phenylmethylsulfonyl fluoride (PMSF), (Roche): 100 mM stock (100×) prepared in methanol or ethanol and stored at  $4^{\circ}\text{C}$ .
3. Tissue sample grinding kit (GE Healthcare Amersham Biosciences) (*see Note 2*).

## 2.2 2-Dimensional Gel Electrophoresis

### 2.2.1 Isoelectric Focusing

1. IPG strips of desired pH range (4–7 pH range for brain tissue and 5–8 pH range for spinal cord).
2. Filter wicks.
3. Isoelectric focusing apparatus.
4. Equilibration trays.

### 2.2.2 Equilibration

1. Equilibration buffer base (EQB): 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% sodium dodecyl sulphate (SDS), 20% (v/v) glycerol. Store as 20- and 40-mL aliquots at  $-20^{\circ}\text{C}$ . While aliquoting, the solution should be constantly stirred using a magnetic stirrer. Glycerol will otherwise accumulate at the bottom. Thaw before use and vortex to get a clear solution.
2. Equilibration buffer 1: EQB containing 2% (w/v) DTT. Dissolve few hours before use at room temperature and keep at dark. Working volume is at least 6 mL per gel strip.
3. Equilibration buffer 2: EQB containing 2.5% (w/v) iodoacetamide (Bio -Rad). Dissolve few hours before use at room temperature and keep at dark. Working volume is at least 6 mL per gel strip.

### 2.2.3 SDS PAGE

1. Tris buffers: 1.5 M Tris-HCl, pH 8.8, and 0.5 M Tris-HCl, pH 6.8. Store at room temperature.
2. 10% SDS. A ready made stock of 20% w/v SDS is diluted 1:2 with water and stored at room temperature.
3. Thirty percent acrylamide/bis solution (37.5:1 with 2.6% C) (Genaxxon) (*see Note 3*).
4. *N,N,N,N'*-Tetramethyl-ethylenediamine (TEMED).

5. Ammonium persulfate (APS): Prepare 10% w/v solution in water before use.
6. Water-saturated isobutanol. Shake equal volumes of water and isobutanol in a glass bottle and allow to separate overnight. Use the top layer. Store at room temperature.
7. Agarose overlay buffer: 0.5% (w/v) agarose is dissolved in Tris glycine SDS (TGS) running buffer by boiling in a microwave. Few drops of bromophenol blue are added to the buffer for color. Store at 4°C. Melt in a microwave before use and maintain at 60°C before use.
8. Running buffer (10×): 250 mM Tris, 1.920 M glycine, 1% (w/v) SDS pH 8.3. Store at room temperature.
9. Prestained molecular weight markers.
10. Hinged spacer plates.
11. Gel casting chamber.
12. Gel combs for 2D gels with 1 reference well (Protean plus comb, Bio –Rad).

### 2.3 *ProQ Diamond Staining*

1. Fixing buffer: 10% acetic acid, 50% methanol. Stored at room temperature or prepared before use (*see Note 4*).
2. ProQ Diamond stain (Molecular Probes), store protected from light at 4°C (*see Note 5*).
3. Destain 1: 20% Acetonitrile (ACN) and 50 mM sodium acetate pH 4.0 (*see Note 6*). For stock buffer solution, dissolve 1 M sodium acetate in double distilled water and adjust the pH to 4.0 using fuming hydrochloric acid (HCl) and store at room temperature.
4. Staining trays compatible with methanol

### 2.4 *Colloidal Coomassie Staining*

1. Colloidal solution: 17 mM ammonium sulphate, 2% phosphoric acid and 34% v/v methanol (*see Note 7*).
2. R-250 Brilliant Coomassie, (Sigma).

### 2.5 *Spot Processing*

1. Destain 2: 1:1 solution of 20 mM  $\text{NH}_4\text{HCO}_3$ , pH 8.00, and 100% ACN. Mix equal volumes and leave and store at room temperature.
2. 1 mM  $\text{NH}_4\text{HCO}_3$  pH 8.0 and trypsin (Sequencing grade modified trypsin, Promega). Dissolve trypsin in 1 mM  $\text{NH}_4\text{HCO}_3$  at 1  $\mu\text{g}/\mu\text{L}$  concentration and store in 5- $\mu\text{L}$  aliquots at -20°C.
3. 2% trifluoroacetic acid (TFA), (Merck) and 5% formic acid ( $\text{HCOOH}$ ), (Merck).

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