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TECHNICAL BRIEF

Development of a simplified, economical polyacrylamide gel staining protocol for phosphoproteins

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Pro-Q Diamond (Pro-Q DPS) is a commercially available stain that binds the phosphate moiety of phosphoproteins with high sensitivity and linearity. To conserve consumable costs we demonstrate that threefold diluted Pro-Q DPS offers the same sensitivity and linearity of signal to that obtained with undiluted Pro-Q DPS. The optimal conditions for Pro-Q DPS indicate that fixation, staining, and destaining of gels longer than 1 h, 2 h, and four 30-min incubations, respectively, are not required. The fixation and destaining solutions, but not the threefold diluted Pro-Q DPS, can be re-used without compromising the signal intensity or linear dynamic range. This modified protocol of Pro-Q DPS reduces the cost at least by fourfold, making the stain economically attractive for large-scale analysis of phosphoproteins.

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Protein phosphorylation is a major post-translational modification by which cells transduce cellular signals [1, 2]. Understanding the dynamics of this post-translational modification in response to cellular as well as abiotic and biotic cues can lead to the identification of candidate regulatory proteins (protein kinases) and their substrates. This new area of system biology is loosely referred to as phosphoproteomics [1, 2]. One of advances in this area is the development of a unique fluorescence stain, Pro-Q Diamond phosphoprotein stain (Pro-Q DPS) [3]. Pro-Q DPS binds directly to the phosphate moiety of phosphoproteins with high sensitivity, and is compatible with other stains, such as glycoprotein and total protein stains, and with modern MS [3–5]. Pro-Q DPS has several advantages over previous approaches, particularly radiolabeling, to determine the phosphorylation status of proteins and peptides [5, 6]. Therefore, the availability of Pro-Q DPS has generated interest towards the development of phosphoproteomes for specific tissues, organs, and whole systems. However, Pro-Q DPS is not economically attractive for conducting phosphoproteomics on a proteome-wide scale. Any alleviation in the consumable expense of Pro-Q DPS would help the scientific community to employ this stain to its full potential to characterize phosphoproteins on a global scale. With this aim, we have initiated an investigation to identify limitations on the extended use of this dye. We demonstrate that threefold diluted Pro-Q DPS in deionized water can be used to detect the phosphoproteins with similar sensitivity and linear dynamic range, as detected by Pro-Q DPS without dilution. We also provide a cheaper and simplified protocol for this stain to detect phosphoproteins in 1- and 2-D gels. These practical changes to the previously published and manufacturer's protocols significantly reduce the expense of this breakthrough technology for the end-user.

1- and 2-DE were performed by standard methods, as described previously [7, 8]; a brief description on the methods is provided in the Supplementary methods. 1- and 2-D gels were stained with Pro-Q DPS (Molecular Probes,

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Abbreviations: PeppermintStick standards, PeppermintStick phosphoprotein molecular weight standards; **Pro-Q DPS**, Pro-Q Diamond phosphoprotein stain; **SYPRO-R PGS**, SYPRO Ruby protein gel stain

Eugene, OR, USA; product no. P33301) following either the standard protocol, which is based on the published data [3, 4] and the manufacturer's protocol (as of July 16, 2004; Molecular Probes) or the optimal conditions presented in this study. Trays used in this study for processing 1- and 2-D gels were of sizes 13.75 cm × 13.75 cm × 5.25 cm (GLAD WARE, Oakland, CA, USA) and 26 cm × 20 cm × 10 cm (Catalog number EFRCP 124PCLE, Daigger, Vernon Hills, IL, USA), respectively. Gel fixing, staining, and destaining were performed with constant shaking on an orbital shaker (GeneMate, ISC Bioexpress, USA) at a speed of 35 rpm. Following Pro-Q DPS staining procedure, gels were imaged using an FLA 5000 laser scanner (Fuji Medical Systems, Stamford, CT, USA) with 532-nm excitation and 580-nm bandpass emission filter. Data were collected as 100-μm, 16-bit TIFF files using the Image Gauge Analysis software (Fuji). With this software, fluorescent protein signals in 1-D gels were displayed as dark bands or, in 2-D gels, dark spots. 1- and 2-D gels were quantitated in profile mode using ImageQuant and ImageMaster 2D Platinum software (Amersham Biosciences, NJ, USA), respectively.

The procedure for detecting phosphoproteins in polyacrylamide gels using the Pro-Q DPS was first described in 2003 and updated 1 year later [3, 4]. These two papers differ slightly with respect to the procedure used for the detection of phosphoproteins. For example, Steinberg *et al.* [3] performed a 90-min to overnight fixation of the gels versus overnight fixation only, used by Schulenberg *et al.* [4]. The most recent protocol recommended by the manufacturer (Molecular Probe; as of 16 July 2004) indicates the use of a different composition of fixation solution, namely 50% methanol and 10% acetic acid, rather than 45% methanol and 5% acetic acid used by Steinberg *et al.* [3] and Schulenberg *et al.* [4]. Additional differences with the destaining solution and time period used for washing the gels were also observed. Steinberg *et al.* [3] compared different destaining solutions including 15% 1,2-propanediol in 50 mM sodium acetate (pH 4.0) and 4% ACN in 50 mM sodium acetate (pH 4.0) for a minimum of 3 h, whereas Schulenberg *et al.* [4] carried out three washes for a period of 30–60 min each in 20% ACN in 50 mM sodium acetate, pH 4.0. In this context, it seems that Pro-Q DPS procedure is still undergoing modifications, and it is therefore possible that the published staining procedures for the Pro-Q DPS are not fully optimized.

Sub-optimal staining conditions might cause difficulties in reproducing results from laboratory to laboratory. Importantly, to minimize the cost and to provide optimal conditions for Pro-Q DPS, an improved standard protocol is needed. To this end, we present an improved protocol for Pro-Q DPS based on the published [3, 4] and manufacturer's protocols (Table 1), which has been termed "standard" protocol in this study. The protocol involves four steps: fixation, washing, staining and destaining. The last three steps are performed in the dark (Table 1) to minimize signal quenching. For fixation solution, we have used 50% methanol and 10% acetic acid in this study, because first, this solution has been found to be the best among the various other 1-D gel fixation solutions [9], and second, it has recently been recommended by the manufacturer (Molecular Probes, as of 16 July 2004). It has been reported that destaining solutions containing ACN are superior to other tested destaining solutions, such as 50 mM sodium acetate, pH 4.0, alone [3]. Recently, 20% ACN in 50 mM sodium acetate, pH 4.0, has been used as a destaining solution [6]. In our experience, this solution works well to destain gel-bound nonspecific Pro-Q DPS and reduce destaining time (data not shown). Therefore, we have used 20% ACN in 50 mM sodium acetate, pH 4.0, as destaining solution throughout this study. Destaining was carried out for 2 h in total (four times 30 min each), because we have observed that this time period is sufficient to obtain a low background (data not shown). All data presented in this study are representative of three independent experiments.

Table 1. The standard protocol for Pro-Q DPS is based on the published [3, 4] and manufacturer's protocols (revised on July 16, 2004; Molecular Probes). The last three steps (staining to washing) should be carried out in the dark. The gel sizes of 1-D and 2-D, used in the published [3, 4] and manufacturer's protocols, were 6 cm × 9 cm × 0.75–1 mm and 20 cm × 20 cm × 1 mm, respectively

Step	Solution	Amount 1-D gel (2-D gel)	Time
Fixation	45% methanol, 5% acetic acid [3] 50% methanol, 10% acetic acid [4][a)]	100 ml (500 mL)	3 × min [3][a)] overnight [3, 4][a)]
Washing	Deionized water	100 mL (500 mL)	3 × 10 min
Staining	Pro-Q DPS	50 mL (500 mL)	90–120 min
Destaining	50 mM sodium acetate, (pH 4.0) 15% 1,2-propanediol [3] 50 mM sodium acetate, (pH 4.0) 4% ACN [3] 50 mM sodium acetate, (pH 4.0) 20% ACN [4][a)]	100 ml (500 mL)	3 × 30–60 min
Washing	Deionized water	100 mL (500 mL)	2 × 5 min

a) Manufacturer's protocol.

Since Pro-Q DPS is expensive, we first determined the effect of dilution of Pro-Q DPS on the fluorescence signal and linear dynamic range. The PeppermintStick phosphoprotein molecular weight standards (PeppermintStick standards; Molecular Probes; product no. P33350) were loaded in a twofold serial dilution series ranging from 1000 ng to 2 ng (lanes 1–10 in Fig. 1), separated by 1-DE, and stained with undiluted and water-diluted Pro-Q DPS (Fig. 1). The PeppermintStick standards contain two phosphorylated (ovalbumin and β–casein of 45.0 and 23.6 kDa, respectively) and four nonphosphorylated (β-galactosidase, bovine serum albumin, avidin, and lysozyme of 116.25, 66.2, 18.0, and 14.4 kDa, respectively) proteins. Both the phosphorylated proteins were specifically detected by Pro-Q DPS. Results indicated that the fluorescence intensity of the phosphoprotein signal remained almost unaffected with dilution of Pro-Q DPS up to threefold and decreased by approximately two- and sixfold with further dilutions at five- and tenfold (Fig. 1A–E). Notably, dilution of Pro-Q DPS up to tenfold did not change the detection limit of these phosphoproteins. The linear dynamic range was also found to be nearly the same up to a threefold dilution, but was compromised to a great extent with five- and tenfold dilution (Fig. 1F). Therefore, threefold diluted Pro-Q DPS can be used to detect phosphoproteins without compromising the sensitivity, fluorescence intensity, or linear dynamic range.

To determine whether prolonged staining time increases the fluorescence intensity and linear dynamic range, gels were stained with threefold diluted Pro-Q DPS for 2, 6, and 12 h (Supplementary Fig. 1). No significant increase in the fluorescence intensity or linear dynamic relationship between fluorescence signal and protein amount was observed even after staining for 12 h (Supplementary Fig. 1A–D). So, the prolonged staining does not improve binding of Pro-Q DPS to phosphoproteins. Therefore, staining of gels longer than 2 h is not required. One should expect this result considering the fact that Pro-Q DPS binds exclusively to the phosphate moiety, not to whole phosphoproteins [3]. In contrast to Pro-Q DPS, total protein stains require longer incubation times to achieve a linear relationship between signal intensity and protein amount. For example, a band containing 1000 ng protein needs more than 7 h to fill all possible binding sites for the fluorescent SYPRO-R PGS [9]. After establishing optimal dilution and staining time for Pro-Q DPS, we used threefold diluted Pro-Q DPS and 2 h of staining in all subsequent experiments, unless otherwise stated. Furthermore, a time course experiment (1, 6, and 15 h) with fixation solution indicated that 1 h is sufficient to fix the gels (Supplementary Fig. 2).

After optimizing the conditions for fixation, staining, and destaining (Table 2), we proceeded to compare phosphoprotein detection using the standard (Table 1) and modified (Table 2) protocols. As shown in Fig. 2A and B, these two images are near identical with the same detection limit for the phosphoproteins (Fig. 2A, B). We also compared the fluorescence intensity and linear range for both the phosphoprotein signals (Fig. 2C, D). Both ovalbumin (45.0 kDa; Fig. 2C) and β–casein (23.6 kDa; Fig. 2D) showed almost the

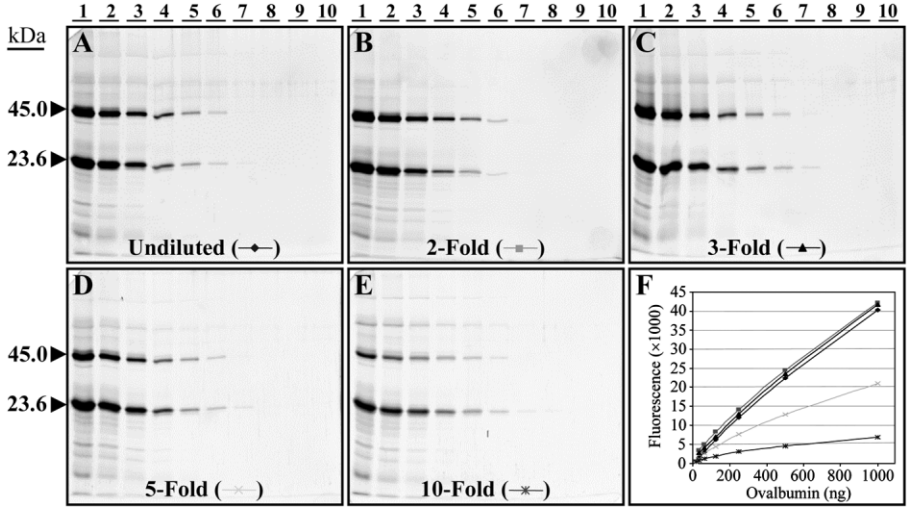

Figure 1. Effect of Pro-Q DPS dilution on signal intensity, detection sensitivity, and linear dynamic range. All gels were fixed (50% methanol and 10% acetic acid), washed two times with deionized water, stained with Pro-Q DPS without (A) or after dilution in deionized water for 2 h (B–E), and destained four times for 30 min with destaining solution (20% ACN in 50 mM sodium acetate, pH 4.0). All gels contain a twofold dilution series of PeppermintStick standards of 1000 ng to 2 ng (lanes 1–10), which were separated by 1-DE (12% SDS-PAGE). All gel images were captured with the same grayscale setting (100 μm, 16-bit) and file format (TIFF). The fluorescence intensity of ovalbumin (45.0 kDa) phosphoprotein bands was quantitated and plotted in (F). Results presented here are representative of three independent experiments.

Table 2. A modified protocol for phosphoprotein detection in polyacrylamide gel using Pro-Q DPS. Steps, staining to washing, should be carried out in the dark. The gel sizes were 8 cm × 8 cm × 1 mm and 26 cm × 20 cm × 1 mm for 1- and 2-D gels, respectively

Step	Solution	Amount 1-D gel (2-D gel)	Time
Fixation	50% methanol, 10% acetic acid[a)]	100 mL (250 mL)	2 × 30 min
Washing	Deionized water	100 mL (250 mL)	2 × 15 min
Staining	"3-fold diluted" Pro-Q DPS in deionized water	65 mL (150 mL)	120 min
Destaining	50 mM sodium acetate, (pH 4.0) 20% ACN[a)]	100 mL (250 mL)	4 × 30 min
Washing	Deionized water	100 mL (250 mL)	2 × 5 min

a) Can re-use.

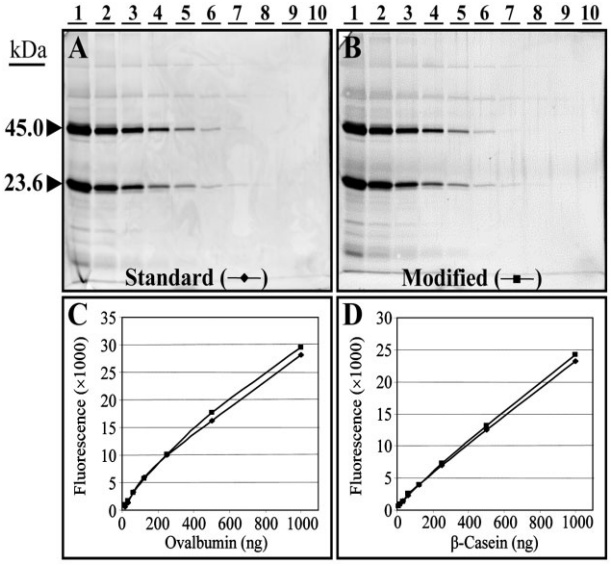

Figure 2. Comparison between the standard and modified protocols for detecting phosphoproteins by 1-DE. Gels were processed according to the conditions mentioned in the Table 1 (A) or as modified (Table 2; fixation for 1 h, staining with threefold diluted Pro-Q DPS for 2 h) in this study (B). The ovalbumin (45.0 kDa) and β-casein (23.6 kDa) phosphoprotein bands were separately quantitated and plotted in (C) and (D), respectively. All other conditions are the same as mentioned in Fig. 1.

same profile with respect to the signal intensity and linear dynamic relationship between the standard and modified protocols. Effectiveness of the modified protocol in detecting phosphoproteins was further demonstrated in 1- and 2-D gels with total proteins extracted from developing seeds (5 weeks after flowering) of *B. napus* plant. The high-resolution 2-D gels stained with modified and standard protocols are given as Supplementary Fig. 3; the modified protocol for 2-D gel (26 cm × 20 cm × 1 mm; DALT, Protean Plus gels) is summarized in Table 2. Repeated experiments indicated that the modified protocol has several advantages over the standard protocol. First, gels stained with the modified protocol show lower background and more distinct detection of very low abundance phosphoproteins. Second, the modified protocol (Table 2) not only reduces the cost of Pro-Q DPS by threefold (directly by threefold dilution of stock), but also by reducing the manufacturer's recommended volume of staining solution for one 2-D gel by more than threefold and other solutions (such as fixation and destaining) in half.

In this final set of experiments, we used the modified protocol (Table 2) to evaluate the effect of "re-use of solutions" (fixation, staining, and destaining) on signal intensity and linear dynamic range (Supplementary Fig. 4). We define re-use of solution "as a solution that has been used one-time and reserved for subsequent experiment". The signal intensity and linear dynamic range for ovalbumin (45.0 kDa) in Supplementary Fig. 4A–E are shown in Supplementary Fig. 4F. It was noticed that gels without a fixation step show a significant decrease in the signal intensity and linear dynamic range (Supplementary Fig. 4B, F), indicating the importance of gel fixation. For checking the feasibility of re-using the solutions, one-time used fixation, staining, and destaining solutions were re-used (Supplementary Fig. 4C–E). It was found that images (Supplementary Fig. 4C, E) obtained with the re-used fixation and destaining solutions are similar to that obtained with the modified protocol (Supplementary Fig. 4A), indicating that these solutions can be re-used at least once. However, re-using the staining solution considerably affected the signal intensity and linear dynamic range (Supplementary Fig. 4D, F). The signal intensity dropped by approximately 28%, from 25 000 to around 18 000 arbitrary units. Careful analysis of images also revealed that the limit of detection of phosphoproteins is not affected to a great extent by re-use of staining solution (Supplementary Fig. 4A–E). It is therefore possible to re-use the staining solution for qualitative detection but perhaps not quantitative analysis of phosphoproteins.

In conclusion, we demonstrate that a threefold diluted Pro-Q DPS produces the same phosphoprotein profiles as seen with undiluted Pro-Q DPS. Optimization of all the

steps involved during use of the threefold Pro-Q DPS has resulted in a modified protocol for the Pro-Q DPS, which is presented here. Importantly, this modified protocol significantly reduces the overall cost of phosphoprotein detection, thereby making Pro-Q DPS a more economically suitable technology for conducting large-scale phosphoproteomics.

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