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Research Article

Increasing the amount of phosphoric acid enhances the suitability of Bradford assay for proteomic research

The Bradford assay is one of the most commonly used methods for protein quantification. However, in proteomic research, the lysis buffer generally used for dissolving proteins can cause some interference to the assay. The dye reagent of classical Bradford assay contains 8.50% (w/v) phosphoric acid, which is an important factor relating to the color yield of the assay. In this study, the phosphoric acid content in dye reagent was increased to 9.35% (w/v), 10.20% (w/v), and 11.05% (w/v) to evaluate the changes of interference and the effects of lysis buffer on the interaction between proteins and dye reagent. Results show that lysis buffer not only causes background interference but also affects the protein–dye chromogenic process. Analysis of different components in the lysis buffer showed that carrier ampholyte is the main factor that introduces interference to the Bradford assay. Detergents are well-known interfering compounds in the Bradford assay, but CHAPS and octyl b-D-glucopyranoside only cause slight interference. When the amount of phosphoric acid was increased from 8.50%(w/v) to 10.20% (w/v), the sensitivity of the Bradford assay to proteins in lysis buffer was increased, and the interference delivered by lysis buffer was considerably reduced.

Keywords:
Bradford assay / Carrier ampholyte / Lysis buffer / Phosphoric acid / Two-dimensional electrophoresis

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1 Introduction

Protein quantification is important for unbiased comparison of electrophoretograms in comparative proteomic analysis. Many methods are available for protein quantification [1, 2], including Kjeldahl method [3], ultraviolet absorption method [4], Biüret assay [5], Lowry assay [6], bicinchoninic acid assay [7], and Bradford assay [8]. Among these methods, the Bradford assay is simple, rapid, cheap, sensitive, and comparably compatible with the lysis buffer, which is widely used in 2DE experiments for dissolving proteins [9–12]. Additionally, a modified Lowry method from Peterson is compatible with the lysis buffer for 2DE [13].

Although the Bradford assay has some advantage over other colorimetric protein assays in 2DE experiments, the interfering effects of lysis buffer cannot be ignored. Lysis buffer must contain large amounts of detergents, carrier ampholytes, and urea to increase the solubility of proteins. These compounds can interact with the Coomassie dye and produce a remarkable background interference to the quantification process [10]. Numerous research works on the interference of lysis buffer have been conducted [14–16], but only a few focus on the reduction of the interference.

The Bradford assay is based on the binding of CBB G-250 to proteins, which results in a protein–dye complex with increased molar absorbance at 595 nm [8]. The dye reagent in the original method comprises 0.01% (w/v) CBB G-250, 4.75% (v/v) ethanol, and 8.50% (w/v) phosphoric acid. Among these components, phosphoric acid is a major factor because it regulates the protonation degree of the CBB G-250 molecules [17]. Insufficient phosphoric acid results in large numbers of unprotonated dye molecules, whereas an excess of phosphoric acid complicates equilibrium displacement due to the excess protons [18]. Some researchers have found that decreasing the phosphoric acid concentration could increase the absorbance and decrease the variability in the chromogenic responses of different proteins [17, 19, 20].

In routine protein quantitation processes, increasing the content of phosphoric acid in the dye reagent was found to lower the interference of lysis buffer. Therefore, the phenomenon was systematically investigated by augmenting the content of phosphoric acid to different proportions. The result demonstrates that the increase in phosphoric acid not only reduces the interference of lysis buffer but also increases the

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Abbreviations: **DDM**, n-Dodecyl β-D-maltoside; **GE**, General Electric; **NP-40**, Nonidet P-40; **OGP**, octyl b-D-glucopyranoside

sensitivity of the Bradford assay to samples containing lysis buffer, making the Bradford assay more compatible with 2DE experiments. In addition, the interfering effect of different kinds of carrier ampholytes and detergents was analyzed.

2 Materials and methods

2.1 Main reagents and equipment

BSA and γ-globulin were obtained from Roche Applied Science (Roche Diagnostics (Shanghai) Limited, Shanghai, China). Ovalbumin, urea, thiourea, octyl b-D-glucopyranoside (OGP), n-Dodecyl β-D-maltoside (DDM), CBB G-250, Triton X-100, DTT, Nonidet P-40 (NP-40), and CHAPS were purchased from Sigma-Aldrich (Sigma-Aldrich Shanghai Trading Co Ltd, Shanghai, China). Pharmalyte (pH 3–10 and pH 5–8), ampholine (pH 3.5–10 and pH 4–6), and IPG buffer (pH 4–7) were obtained from GE Healthcare (General Electric Co. (China), Shanghai, China). All chemicals used were of analytical grade or the best grade available. The H_2O used in this paper refers to Milli-Q water.

2.1.1 Dye reagent

CBB G-250 (100 mg) was dissolved in 50 mL 95% ethanol. A total of 100, 110, 120, or 130 mL of 85% (w/v) phosphoric acid was added to this solution. The resulting solution was diluted to a final volume of 1 L with H_2O and immediately filtered. The dye reagent was stored in brown bottles. For convenience, the dye reagent with 100, 110, 120, or 130 mL of 85% (w/v) phosphoric acid per liter was designated as Dye (8.50%), Dye (9.35%), Dye (10.20%), and Dye (11.05%), respectively.

2.1.2 Lysis buffer

The standard urea/thiourea lysis buffer [21], which contains 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (w/v) DTT, and 2% (v/v) pharmalyte (pH 3–10), was used. The buffer was aliquoted into Eppendorf tubes (1 mL/tube) and stored at −20°C before use.

2.1.3 Standard solutions of proteins

Three kinds of protein solutions (BSA, ovalbumin, and γ-globulin) were used as standard, all at a concentration of 1 mg/mL in H_2O. The protein concentration in the standard solutions was determined spectrophotometrically based on the absorbance at 280 nm of 1 mg/mL solution of BSA, ovalbumin, and γ-globulin in 1 cm light path, resulting in 0.66, 0.75, and 1.35, respectively [22]. These solutions were also aliquoted and stored frozen at −20°C.

The absorbance of all samples was measured using a Shimadzu dual beam path spectrophotometer UV-1800 (Shimadzu Corp., Kyoto, Japan).

2.2 Methods

2.2.1 Measurement of the interaction between lysis buffer and different dye reagents

Certain amounts of lysis buffer (20, 40, 60, 80, and 100 μL) were pipetted into test tubes (15 × 100 mm, the same below). The volumes in the test tubes were adjusted to 100 μL with H_2O. Then, 5 mL of Dye (8.50%) was added to each test tube and were mixed by shaking. After incubation for 5 min, the absorbance at 595 nm (OD595nm) was measured in 3 mL cuvettes against a reagent blank prepared from 100 μL of H_2O and 5 mL of Dye (8.50%). The same process was repeated with the three other kinds of dye reagents.

2.2.2 Sensitivity evaluation of the Bradford assay to standard proteins with or without lysis buffer

Solutions of BSA at the concentrations of 0.20, 0.40, 0.60, 0.80, and 1.00 mg/mL were prepared, and 100 μL of each of the preceding solutions was added to a test tube with 5 mL of Dye (8.50%). After 5 min, OD595nm was measured in 3 mL cuvettes against a reagent blank prepared from 100 μL of H_2O and 5 mL of Dye (8.50%). When evaluating the effect of lysis buffer on the interaction between the dye and proteins, the same process as above was performed, except for the addition of 100 μL of lysis buffer to the reagent blank and the standard protein sample group. The same performance was repeated using the two other kinds of standard proteins (ovalbumin and γ-globulin) and the three other kinds of dye reagents (Dye (9.35%), Dye (10.20%), and Dye (11.05%)).

2.2.3 Evaluation of the interference of the individual components in lysis buffer

Solutions containing only one component of the lysis buffer were prepared in H_2O. The concentrations of individual components were the same as those in the lysis buffer. Sample volumes were all 100 μL, and the measurement process was the same as indicated above.

3 Results and discussion

3.1 Effect of phosphoric acid on the interference of lysis buffer

Lysis buffer contains large amounts of interfering substances, and at least one of these compounds interferes with the Bradford assay [22]. Phosphoric acid is a key component in the dye reagent of the Bradford assay [17,19,20]. The relationship between the interference of lysis buffer and the content of phosphoric acid in the dye reagent has not been studied. Results shown in Fig. 1 demonstrate that the increase in phosphoric acid concentration in the dye reagent could markedly reduce

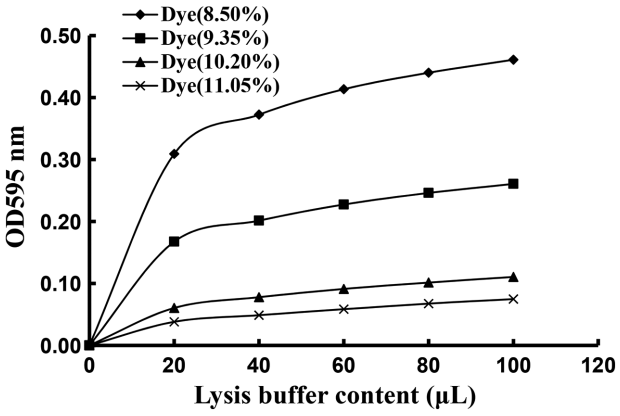

Figure 1. Interaction of different amounts of lysis buffer with dye reagents containing different amounts of phosphoric acid. (A) Sample volumes were all 100 μL. (B) Different volumes of lysis buffer were adjusted to 100 μL with H_2O. (C) Absorbance at 595 nm (OD595nm) was measured in 3 mL cuvettes against a reagent blank prepared from 100 μL of H_2O and 5 mL of corresponding dye reagent. d) The values represented the $\bar{x} \pm s$ of OD595nm for three replicate samples.

the OD595nm of lysis buffer. When using Dye (10.20%) or Dye (11.05%), the interference of lysis buffer drops to only 20 or 13% of that using the Dye (8.50%), respectively, indicating that the increase in phosphoric acid improves the compatibility of the Bradford assay with chemicals in the lysis buffer.

3.2 Effect of phosphoric acid on the sensitivity of the Bradford assay

Sensitivity to proteins is another important factor that must be considered for evaluating a dye reagent in protein quantification. The sensitivity of different dye reagents to various kinds of standard proteins without the interference of lysis buffer is first examined. As shown in Fig. 2, with the increase in the content of phosphoric acid, the sensitivity of the Bradford assay decreases with all three kinds of standard proteins. When phosphoric acid constitutes 11.05% of the dye reagent, the absorbance for all three standard proteins drops to approximately half of the value obtained with the original dye reagent Dye (8.50%). Guo [23] also reported that the sensitivity for BSA decreases as the concentration of phosphoric acid in the dye reagent increases from 6.80 to 13.60%.

When lysis buffer is added to the protein–dye system, with the increase in phosphoric acid content in the dye reagent from 8.50% to 11.05%, the OD595nm for all three standard proteins first rises then drops, as illustrated in Fig. 3, which is quite contrary to the patterns in Fig. 2. The dye reagent containing 10.20% of phosphoric acid shows the highest OD595nm, which indicates the highest sensitivity to proteins. The result from Figs. 2 and 3 indicates that lysis buffer not only introduces some background absorbance reading but also affects the complex formation between CBB G-250 and proteins.

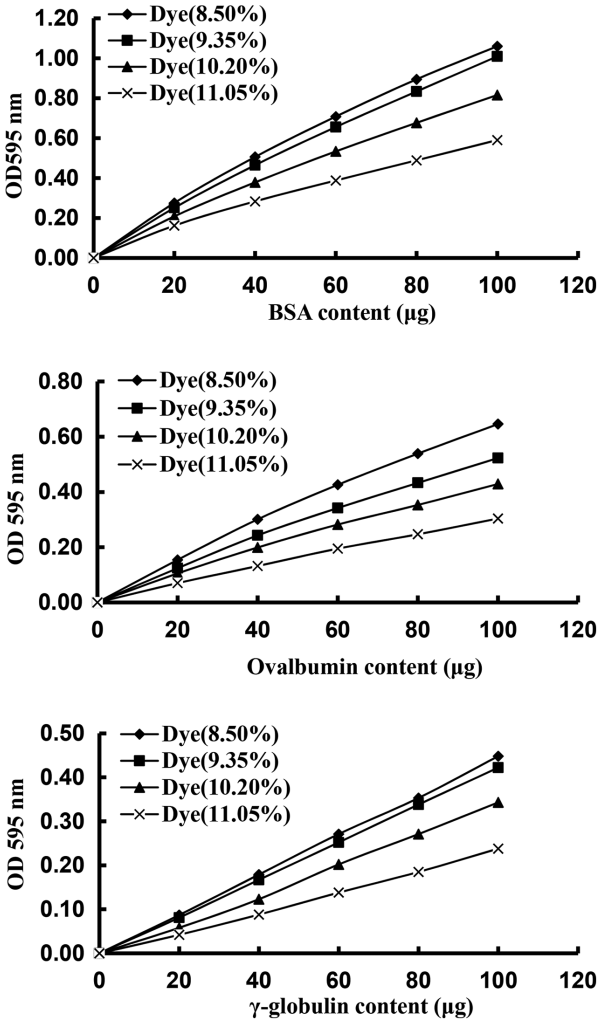

Figure 2. Interaction of different standard proteins with dye reagents containing different amounts of phosphoric acid without the interference of lysis buffer. (A) Sample volumes were all 100 μL. (B) Absorbance at 595 nm (OD595nm) was measured in 3 mL cuvettes against a reagent blank prepared from 100 μL of H_2O and 5 mL of corresponding dye reagent. (C) The values represented the $\bar{x} \pm s$ of OD595nm for three replicate samples.

Notably, when interacting with a certain kind of dye reagent, the OD595nm of different standard proteins at a certain concentration is quite different. BSA is extensively used as a standard in protein quantification because it is cheap and readily available in a pure form. The two other commonly used standards are ovalbumin and γ-globulin. The results illustrated in Figs. 2 and 3 show that all the three standard proteins display similar trends in the change of sensitivity with the increase in phosphoric acid in the dye reagent in the absence or presence of lysis buffer. However, a large difference in the sensitivity exists among different standard proteins, with BSA as the most sensitive and γ-globulin with the least sensitivity. Several studies have reported the importance of lysine and arginine residues in the binding of CBB G-250 to proteins [24–27]. The diverse amino acid composition may be the cause of different sensitivities among BSA, ovalbumin,

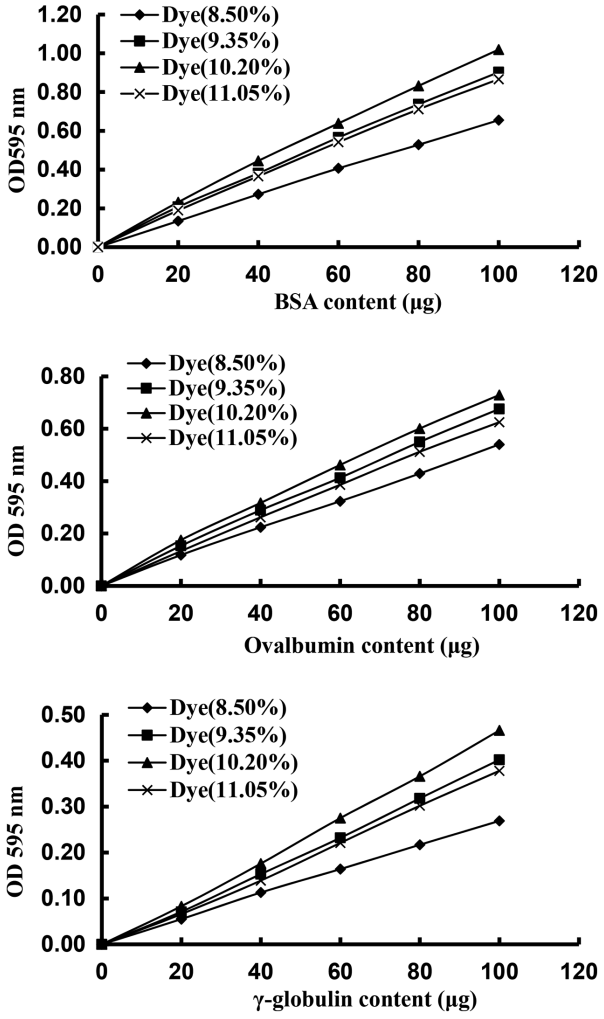

Figure 3. Effect of lysis buffer on the interaction of different standard proteins with dye reagents containing different amounts of phosphoric acid. (A) Sample volumes were all 100 μL. (B) Lysis buffer (100 μL) was added to the standard protein samples and the reagent blank. (C) Absorbance at 595 nm (OD595nm) was measured in 3 mL cuvettes against a reagent blank prepared from 100 μL of H_2O, 100 μL of lysis buffer, and 5 mL of corresponding dye reagent. (D) The values represented the $\bar{x} \pm s$ of OD595nm for three replicate samples.

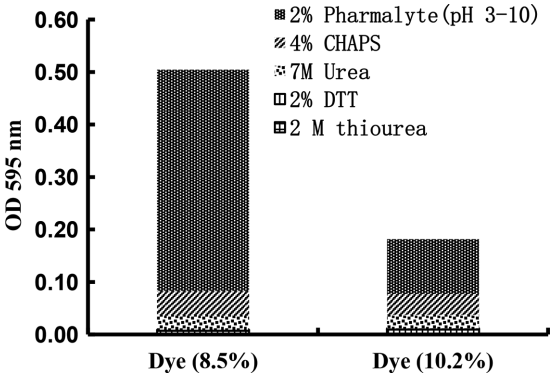

Figure 4. Interference of different components in the lysis buffer to the Bradford assay. (A) Sample volumes were all 100 μL. (B) The concentrations of individual components were the same as those in the lysis buffer. (C) Absorbance at 595 nm (OD595nm) was measured in 3 mL cuvettes against a reagent blank prepared from 100 μL of H_2O and 5 mL of corresponding dye reagent. (D) The values represented the $\bar{x} \pm s$ of OD595nm for three replicate samples.

and γ-globulin. Accordingly, providing a clear indication of the standard proteins used, which was not performed in most related literature, is essential.

One of the functions of lysis buffer is the denaturation of proteins, which extends the structure of proteins and exposes amino acids, completing the complex formation between CBB G-250 and proteins. This phenomenon may be the reason for the enhanced sensitivity of proteins due to lysis buffer; however, the exact mechanism must still be further researched. The theory [18] that excessive amount of phosphoric acid, which supplies an excess of protons and disturbs the equilibrium displacement, may be an appropriate explanation for the lower sensitivity in Dye (11.05%) than that in Dye (10.20%) in Fig. 3.

As shown in Figs. 2 and 3, regardless of what kind of standard protein is used, the OD595nm of the same amount of protein without the effect of lysis buffer is remarkably different from that with lysis buffer. The result suggests that the effect of lysis buffer should be considered when constructing a standard curve for quantitating protein samples containing lysis buffer; otherwise, the measured value will have a large deviation.

3.3 Interference of individual components in lysis buffer

Denaturants, detergents, reductants, or other chemicals are generally utilized in 2DE experiments for denaturing proteins or enhancing their solubility [10]. The aforementioned results demonstrate that Dye (10.20%) has a considerable advantage in quantitating 2DE samples because it can lower the interference of lysis buffer and increase the sensitivity of the Bradford assay. However, the compatibility of Dye (10.20%) with all or only some of the constituents in the lysis buffer remains unknown. Therefore, the interference of individual components of the lysis buffer was studied using Dye (8.50%) and Dye (10.20%).

Figure 4 shows that the interference caused by 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, or 2% (w/v) DTT is little and does not change much in Dye (8.50%) and Dye (10.20%). By contrast, the interference of 2% (v/v) pharmalyte (pH 3–10) remarkably reduces with the increase in phosphoric acid in the dye reagent.

According to the instructions of the manufacturer (e.g., Handbook 80-6429-60AC, GE Healthcare), in 2DE experiments, protein samples loaded onto IPG strips should be prepared in lysis buffer containing various kinds of carrier ampholytes (pharmalyte reagents, ampholines, or IPG buffers). Pharmalyte (pH 5–8), ampholine (pH 3.5–10),

Table 1. Interference of different carrier ampholytes and detergents to the Bradford assay

Category	Reagent name	Absorbance at 595 nm	
		Dye (8.50%)	Dye (10.20%)
2% (v/v) carrier ampholytes	Ampholine (pH 3.5–10)	0.126 ± 0.004	0.011 ± 0.001
	Ampholine (pH 4–6)	0.113 ± 0.004	0.010 ± 0.001
	Pharmalyte (pH 3–10)	0.421 ± 0.004	0.104 ± 0.002
	Pharmalyte (pH 5–8)	0.479 ± 0.005	0.122 ± 0.002
	IPG buffer (pH 4–7)	0.425 ± 0.003	0.083 ± 0.003
4% (v/v) detergents	CHAPS	0.047 ± 0.002	0.040 ± 0.001
	Triton X-100	2.466 ± 0.007	2.502 ± 0.006
	NP-40	2.456 ± 0.004	2.486 ± 0.004
	OGP	0.009 ± 0.002	0.015 ± 0.002
	DDM	1.736 ± 0.003	1.498 ± 0.004

a) Concentrations of different kinds of carrier ampholytes and detergents were the same as those in the lysis buffer.
b) Sample volumes were all 100 μL.
c) Absorbance at 595 nm was measured in 3 mL cuvettes against a reagent blank prepared from 100 μL of H_2O and 5 mL of the corresponding dye reagent.
d) The values represented the $\bar{x} \pm s$ of OD595nm for three replicate samples.

ampholine (pH 4–6), and IPG buffer (pH 4–7) were also examined to check whether some other kinds of carrier ampholytes have a similar response as that of pharmalyte (pH 3–10). As expected, the result showed that these carrier ampholytes have similar response as pharmalyte (pH 3–10) (Table 1), that is, Dye (10.20%) is more compatible with these carrier ampholytes than Dye (8.50%).

The data in Table 1 also illustrated that interference effects of ampholine (either pH 3.5–10 or pH 4–6) are much smaller than those of pharmalyte (either pH 3–10 or pH 5–8). Carrier ampholytes are a heterogeneous mixture of synthetic polymers incorporating a variety of acidic and basic buffering groups. The net charges of ampholyte molecules partly depend on the pH of the environment [28]. When the concentration of phosphoric acid increases, the dye reagent becomes more acidic, and more ampholyte molecules have a net positive charge. The interference of carrier ampholytes is reduced probably because ampholyte molecules with a net positive charge do not easily bind with dye molecules.

The incompatibility of detergent- or surfactant-containing samples with the Bradford assay is commonly recognized [2]. Bradford reported that 0.1 mL of 1% Triton X-100 or 1% SDS could present abnormalities that are difficult to overcome [8]. Non-ionic detergents, such as Triton X-100, NP-40, and DDM, are found to have interfered severely with the assay using either kind of dye reagent (Table 1), which is consistent with what Bradford found. OGP is also a non-ionic detergent applied to isoelectric focusing and 2DE [29] but has almost no interfering effect on any dye reagent. The interfering effect of CHAPS, a kind of zwitterionic detergent, is also small. When quantitating 2DE samples with the Bradford assay, selecting proper carrier ampholytes and detergents, which can meet requirements for research and also interfere as little as possible, is better to obtain accurate quantification results.

4 Concluding remarks

Based on the aforementioned results, the optimized dye reagent recipe for 2DE samples is as follows: 0.01% (w/v) CBB G-250, 4.75% (v/v) ethanol, and 10.20% (w/v) phosphoric acid. The dye reagent is made by dissolving 100 mg of CBB G250 in 50 mL of 95% (v/v) ethanol. The solution is then mixed with 120 mL of 85% (w/v) phosphoric acid and made up to 1 L with distilled water. Then, the reagent should be filtered and stored in an amber bottle at room temperature, remaining stable for several weeks. The alternative quantification protocol contains three parts. First, a standard curve in the range of 0–100 μg standard protein is constructed. The addition of 100 μL of lysis buffer to the standard protein samples and the reagent blank must be ensured. Second, practical 2DE samples are quantitated by pipetting 100 μL of practical sample and 5 mL of dye reagent to a test tube and mixing well by inversion or shaking. After incubation for 5 min, the absorbance at 595 nm of the mixtures can be measured against the reagent blank prepared from 100 μL of lysis buffer and 5 mL of dye reagent. Finally, the protein concentration is calculated according to the standard curve. If the calculated protein concentration exceeds 1 μg/μL, then assay is performed for a range of dilutions.

Overall, by increasing the amount of phosphoric acid in the dye reagent from 8.50 to 10.20% (w/v), the interference of lysis buffer is effectively reduced, and the relatively highest sensitivity of the Bradford assay is realized in quantitating 2DE samples, making the Bradford assay more compatible with 2DE experiments. The decrease in the interference caused by carrier ampholytes is the main reason why increasing the proportion of phosphoric acid can evidently reduce the interference of lysis buffer. The selection of proper standard protein and less interfering components and the addition of lysis buffer in the construction of a standard curve are important factors to be considered for an accurate 2DE protein sample quantification, which is essential for subsequent electrophoretogram analysis and loading quantity references among different researchers and laboratories.

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