Simultaneous Detection of Phosphoproteins and Total Proteins in SDS-PAGE Using Calcon

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A novel fluorescent staining protocol to detect phosphoproteins in sodium dodecyl sulfate-polyacrylamide gels using a fluorescence sensor, 1-(2-hydroxy-1-naphthylazo)-2-naphthol-4-sulfonic acid sodium salt (Calcon), was developed. This method yields results within 135 min, with the sensitivities of 15 ng of α-casein and β-casein, and 62.5 ng of κ-casein, respectively. Since non-phosphoproteins have shown negative signals that are distinctly different from positive signals of phosphoproteins, this detection method allows one to monitor phosphoproteins with high specificity. Furthermore, a total protein profile can be achieved before a destaining step using a scanner with rapid and low-cost without further total protein staining.

Keywords Aluminum, Calcon, fluorescent detection, phosphoprotein stain, Pro-Q diamond, SDS-PAGE

(Received July 2, 2018; Accepted August 29, 2018; Advance Publication Released Online by J-STAGE September 14, 2018)

Introduction

The phosphorylation of proteins plays pivotal roles in regulating the fundamental cellular functions in eukaryotic organisms as well as signaling pathways, leading to many cellular activities. Therefore, the analysis of protein phosphorylation has become an important issue in comprehensive proteomic studies.

Among the present strategies for proteomic research, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most widely used techniques for the separation and identification of the proteins from complex mixtures. Based on this approach, phosphoprotein profiles in gel can be obtained with staining methods, such as Stains-All and Pro-Q Diamond (Pro-Q).

Stains-All is a cationic carbocyanine dye used to visualize nucleic acid that is stained blue and protein that is stained red. And it has been utilized for the determination of some phosphoproteins stained blue. Unfortunately, this method has drawbacks, including generally unacceptable sensitivity and specificity for phosphoproteins with a long staining/destaining time, and a lack of stability to light.

One of the most common detection methods for phosphoprotein in SDS-PAGE is Pro-Q stain using a particular fluorescent dye. It provides an efficient approach to detect phosphoprotein with high sensitivity and good linearity to facilitate both the qualitative and quantitative analysis. Furthermore, Pro-Q is capable of sequentially detecting the expression levels of total proteins in a single gel using a fluorescent total protein indicator (SYPRO Ruby). However, Pro-Q also has disadvantages, such as a relatively low specificity for phosphoproteins, high cost, time-consuming and a complicated procedure.

In our previous studies, several fluorescent detection methods using Alizarin Red S, Morin Hydrate, Fura 2 pentapotassium salt, 8-guonolinol and anthracene chrome Red A were introduced for phosphoprotein detection in gel. However, these stains also showed some limitations in the selectivity or sensitivity.

In order to overcome a lack of detection methods that did not satisfy all of the requirements, new approaches to detect phosphoproteins in SDS-PAGE should be not only sensitive and accurate, but also speedy, convenient and cost-effective.

In this study, a fluorescent probe, which is termed 1-(2-hydroxy-1-naphthylazo)-2-naphthol-4-sulfonic acid sodium salt (known as Calcon or Eriochrome blue black R), has been applied for phosphoprotein detection. It is a widely used anionic dye as an indicator for the EDTA titrations of a variety of metals. Figure 1 shows the chemical structure of this fluorescent probe. An azo chromophore (-N=N-) and two aromatic hydroxyl groups in the structure give rise to not only electrostatic interactions, but also stability between the dye and the trivalent metal. On the other hand, through a specific binding affinity of positively charged metal ions such as Al, Fe, Ga to the phosphate group in phosphopeptide or phosphoproteins. In the present study, Al plays a crucial role for the selective recognition of phosphoproteins as a binding bridge between Calcon and the phosphate groups in phosphoproteins. Since non-phosphoproteins result in negative signals that are distinctly different from positive signals of phosphoproteins, total proteins are able to be detected simultaneously without further staining. Furthermore, a colored total protein profile can also be achieved before a destaining step using a scanner. This approach allows for highly specific, low-cost, rapid, simple and simultaneous detections of phosphoproteins and total proteins on SDS-PAGE.

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Experimental

Materials
Acrylamide, Bis, TEMED, ammonium persulfate (APS), Tris base, glycine, SDS, iodoacetamide, glycerol, bromophenol blue, phosphorylase b (no phosphate), bovine serum albumin (BSA, no phosphate), ovalbumin (OVA, 2 phosphates), glyceraldehyde-3-phosphate dehydrogenase (no phosphate), α-casein (7 or 8 phosphates), β-casein (5 phosphates), κ-casein (1 phosphate), avidin (no phosphate), Calcon (Cat #45550), Stains-All, CBBR and alkaline phosphatase were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). CHAPS, DTT, urea, IPG strip, cover oil and IPG buffer were from GE Healthcare™ (Uppsala, Sweden). Pro-Q Stain, Pro-Q Destain and SYPRO Ruby Gel Stain were from Invitrogen™ (Carlsbad, CA, USA). All other chemicals used were of analytical grade and were obtained from various commercial sources.

Solution preparation
Calcon was dissolved in 40% methanol (MeOH) at a concentration of 1 mM as a stock solution and covered with foil to block light. Stock solutions of the Al$_2$(SO$_4$)$_3$·H$_2$O (10 mM) concentration of 1 mM as a stock solution and covered with foil and sodium acetate trihydrate (SA) (3 M) were prepared with deionized water (DW), respectively, and could be stored for a few weeks. In each case, the working solution was prepared just before use.

Dephosphorylation of protein sample
The phosphoproteins were dephosphorylated according to the protocol using alkaline phosphatase. Briefly, 4 μL of the protein standard mixture (BSA, OVA, α-casein and β-casein ≈ 2 mg/mL proteins) were treated with 100 U of alkaline phosphatase and 1.6 μL of a 10× dephosphorylation buffer (50 mM Tris, 100 mM NaCl, 10 mM MgCl$_2$, and 1 mM DTT; pH 7.9) and incubated at 30°C for 1 h. Reactions were stopped by adding 4 μL of a 5× sample buffer and boiling for 5 min.

Protein staining
Calcon stain. After electrophoresis, a gel was fixed twice in 100 mL of 50% ethanol (EtOH), 10% acetic acid (HAc) for 30 min each, and soaked in 50 mL of 150 μM Al$^{3+}$ aqueous solution for 15 min, followed by washing with 20% MeOH for 15 min. The gel was then immered in 50 mL of the staining solution for 30 min. The staining solution was prepared at a concentration of 60 μM Calcon in 40% MeOH by diluting the Calcon stock solution. Before destaining, a colored total proteins image could be obtained by a scanner (V700, Epson, Seiko, Japan). Finally, for phosphoproteins, gel was developed by soaking the gel in 50 mL of 75 mM SA in 30% MeOH for 30 min each, and soaked in 100 mL of 50% ethanol (EtOH), 10% acetic acid (HAc) for 15 min. For the best results, the container was wrapped with aluminum foil to avoid light exposure during the staining and destaining, and all steps were performed under continuous gentle agitation. Since the method presented here is for a mini-gel, the volumes need to be increased for larger gels.

Pro-Q stain. This staining method was conducted according to the instructions of Invitrogen™. Gel was fixed twice for 30 min or overnight in 100 mL of 50% MeOH and 10% HAc solution, and washed with DW for 10 min thrice with gentle agitation. The gel was then incubated with 50 mL of Pro-Q staining solution for 90 min, and destained three times with 100 mL of Pro-Q destaining solution for 30 min each. Finally, the gel was washed twice with DW for 5 min each. The gel was carried out in the dark for the staining and destaining steps with gentle agitation to achieve optimal results.

Stains-All stain. This staining method was adapted from a procedure of Green et al. First, the gel was fixed in 30 mL of 25% isopropanol at 30°C for 15 min to remove SDS, and then placed in the staining solution in the dark overnight by agitation. The working solution was prepared just before use by combining 10 mL of the stock solution (0.1% Stains-All in formamide), 10 mL formamide, 50 mL isopropanol, 1 mL 3.0 M Tris-HCl (pH 8.8) and DW to a volume of 200 mL. Finally, the gel was destained in DW until a good contrast between the bands and background was observed.

CBBR stain. After electrophoresis, in order to obtain a total protein image, a gel was washed in 100 mL of 40% MeOH,
10% HAc for 2 h to ensure adequate removal of SDS. The gel was stained in 50 mL of 0.1% CBBR in 40% MeOH, 10% HAc for 1 h, followed by two consecutive destainings of 40% MeOH, 10% HAc solution for 1 h each. **SYPRO Ruby stain.** This staining method was performed according to the instructions of Invitrogen™. Subsequent to Pro-Q staining, SYPRO Ruby was used to obtain a total protein stained image in the same gel. Pro-Q stained gel was immersed in 50 mL SYPRO Ruby staining solution overnight and rinsed in 10% MeOH, 7% HAc solution for 30 min. Before imaging, the gel was rinsed in DW for 5 min twice. To achieve better results, all staining and washing steps were performed with continuous gentle agitation, and the container was wrapped with aluminum foil to avoid light exposure.

**Image analysis**

To obtain the best sensitivity and selectivity of each stain, several imaging systems including UV transilluminator, luminescent image analyzer LAS-4000 mini (LAS), and Typhoon 9400™ were appropriately selected according to the staining properties. Firstly, determining the proteins on SDS-PAGE stained with Pro-Q and SYPRO Ruby was carried out using a Typhoon 9400™ scanner (Amersham Biosciences). A 500-nm longpass emission filter and a 532-nm laser excitation source were used for Pro-Q, and a 610-nm longpass emission filter and a 532-nm laser excitation source were used for SYPRO Ruby, respectively. Secondly, to obtain the total proteins image with Calcon (before destaining step), Stains-All and CBBR, image acquisition and analysis were performed under a white background using a scanner (V700, Epson). Thirdly, Calcon stained gels (after destaining step) were visualized by LAS with Y515-Di filter using the following parameters: Fluorescence, SYBR Green, Blue light, F 0.85 Iris, EPI digitized mode, 10 s increment, and standard sensitivity/resolution.

**Results and Discussion**

**Optimization of staining condition**

To optimize the staining procedure, numerous conditions using α-casein and BSA as representative phospho- and non-phosphoproteins were conducted by comparing the specificity and sensitivity. After SDS-PAGE, some elements that are from the electrophoresis buffer may interfere with the phosphoprotein determination. Therefore, a fixation with some solvents (such as MeOH, EtOH and HAc) prior to gel staining is required to maximize the sensitivity. HAc in the fixing solution considerably affected the signal intensity and specificity of phosphoproteins. According to the results using a series of fixing solutions containing HAc at different concentrations (2 – 10%), the intensities of the bands decreased with increasing concentrations of HAc in both BSA and α-casein. An important difference is that a slight decrease in the α-casein intensity was observed with high concentrations of HAc, whereas a significantly decreased (i.e., negative) signal of BSA was observed at the same time (Supporting Information Fig. S1). Since significant differences between phosphoproteins and non-phosphoproteins were revealed in higher concentrations of HAc, a series of concentrations of MeOH or EtOH were tested with 10% HAc. It was found that 50% EtOH is the most appropriated solvent for giving sharper bands and better contrast. Therefore, the applicable and suitable fixing solution for the best specificity of Calcon stain was determined to be 50% EtOH with 10% HAc. With regard to the fixing time, an examination in the range from 15 min twice to overnight revealed that 30 min twice was sufficient to obtain optimal sensitivity and specificity. For prolonged periods, it gave almost identical results in terms of the sensitivity, whereas a shorter fixing time resulted in a comparatively poor specificity for the determination of phosphoprotein.

It is known that Al³⁺ binds specifically to the phosphate groups of phosphoproteins through electrostatic attraction; therefore, the binding solutions containing various concentrations of Al³⁺ (from 0 to 300 µM) were evaluated. Supporting Information (Fig. S2) shows fluorescence signal changes in the absence or presence of various concentrations of Al³⁺. Incubation without Al³⁺ showed no band signal, whereas the emission of the band exhibited a dramatic increase in the fluorescence intensity of α-casein at 150 µM of Al³⁺. However, decreases in the intensity of α-casein were observed at Al³⁺ concentrations higher than 150 µM. On the other hand, BSA showed relatively weak negative bands after the addition of Al³⁺ in the range of 50 – 200 µM. Therefore, 150 µM Al³⁺ was chosen to increase the specificity for the phosphoproteins.

It was confirmed that washing the gels with various concentrations (0, 10, 20 and 30%) of MeOH to remove excess Al³⁺ from the gel matrix had an influence on the sensitivity. The intensity of α-casein was increased with MeOH in the washing solution, as shown in Supporting Information (Fig. S3). At more than 20% of concentration the intensity was decreased. Therefore, it was decided that 20% MeOH is appropriate to obtain better results.

The staining solution is the key factor for specific phosphoprotein detection. To achieve the optimal staining condition for the best specificity and sensitivity, we explored a series of dye concentrations, ranging from 7.5 to 120 µM (Supporting Information, Fig. S4). The results showed that low concentration of Calcon resulted in low specificity of phosphoproteins. However, some improvements were observed in both of the specificity and sensitivity of α-casein by increasing the concentration of Calcon. As higher concentration than 60 µM of Calcon resulted in a slight decrease in α-casein fluorescence, the optimal concentration of Calcon was found to be 60 µM. For the staining solution in this study, on the other hand, the solvent composition was tested with a series of concentrations ranging from 0 to 40%, and decided by 40% MeOH. To determine whether a prolonged staining time increases the fluorescence intensity, in addition, gels were stained in the staining solution for 15, 30, 60, 120, 240 min and overnight, respectively. The results indicated that there was no significant increase in the sensitivity in a staining time longer than 30 min, but was compromised by a time less than 30 min. Therefore, an optimum staining condition was taken to be 60 µM Calcon in 40% MeOH for 30 min, considering the sensitivity and speed.

SA in the destaining solution is another critical factor to increase the specificity. To determine the optimum SA concentration, SA was tested in varying concentrations from 0 to 100 mM in the destaining solution. The results indicate that the specificity and sensitivity were significantly increased by the addition of SA (Supporting Information Fig. S5). It was therefore decided to develop the image with 75 mM in 30% MeOH for 15 min in the dark. Considering the sensitivity, specificity and speed, the optimal procedure was established up (Fig. 2).

**Protein detection in SDS-PAGE**

To compare the specificity and sensitivity with different stains, two-fold dilution series of eight different protein markers (phosphorylase b, BSA, OVA, glyceraldehyde-3-phosphate
dehydrogenase, α-casein, β-casein, κ-casein, and avidin) were loaded on SDS-polyacrylamide gels ranging from 2 to 1000 ng. After separation by 1-D, the gels were stained with Calcon, Pro-Q and Stains-All to visualize the phosphoproteins, and total proteins were identified using SYPRO Ruby and CBBR stain, respectively. By comparing with the other phosphoprotein detection methods, the sensitivity of Calcon stain toward β-casein (15 ng/band) is less than Pro-Q stain (2 ng/band), but superior to Stains-All (62.5 – 125 ng/band). As shown in Fig. 3A, the sensitivities of Calcon stain were 15 ng of α-β-casein, 62.5 ng of κ-casein and 125 ng of OVA, respectively. Since the specific phosphoprotein detection in Calcon stain is achieved by the cooperative action with the selectivity of Al³⁺ for the phosphate groups in proteins and the affinity of Calcon for Al³⁺, the sensitivity and specificity depend on the phosphorylation level (the number of phosphate groups in protein). The number of phosphates of OVA or κ-casein is 2 or 1, which is much less than those of α-casein or β-casein containing 7 – 8 or 5 phosphates, respectively. According to the proposed mechanism of Calcon, OVA or κ-casein was observed with a relatively weaker signal than α-casein or β-casein after staining, but it clearly differed from the expression of non-phosphoproteins. As shown in Fig. 3B, Pro-Q stained gel also shows that OVA and κ-casein are less sensitive than α-casein or β-casein.

In addition, Calcon stain shows a relatively better specificity, and significantly reduces the operating time (135 min) and associated costs compared to other methods. Stains-All induces a relatively poor specificity, sensitivity and a lack of stability to light.
As shown in Fig. 3C, OVA was stained red with Stains-All, though it was phosphoprotein; furthermore the color of Stains-All began to fade in a few minutes. Although Pro-Q stain is one of the most sensitive commercial stain methods for phosphoproteins, it has been observed that Pro-Q stain detects not only phosphoproteins, but also high abundant non-phosphoproteins. As shown in Fig. 3B, non-phosphoproteins, such as phosphorylase b, BSA and glyceraldehyde-3 phosphate dehydrogenase, were markedly visible in 1000 – 62.5 ng/band by Pro-Q (false positive). In the Calcon stained gel; however, phosphoproteins showed positive bands, while non-phosphoproteins appeared as negative (transparent) signals (Fig. 3A). This allows a high specificity for phosphoproteins due to clear discrimination between phosphoprotein and non-phosphoprotein signals, which reduces false positives for non-phosphoproteins.

To identify total proteins, on the other hand, Pro-Q stain demands labor-intensive sequential stainings (e.g., SYPRO Ruby), which require extra cost and several steps with a long time. Calcon stain is possible to detect total proteins in two ways. First, Calcon stain can simultaneously identify and distinguish between phosphoprotein representing a positive signal and non-phosphoprotein representing a negative signal, even with a single stain (Fig. 3A). Otherwise, it is possible to visualize the pink-colored total proteins before the destaining step in the staining process (Fig. 3E), with similar sensitivity of the CBBR stain (Fig. 3F).

In order to demonstrate the specificity of the Calcon stain, a novel method was performed in the detection of phosphoproteins on 2-D SDS-PAGE using non-treated and phosphatase-treated phosphoprotein standards (Fig. 4). After Calcon staining of non-treated standard markers, phosphoprotein spots (OVA, α-casein and β-casein) and non-phosphoprotein spots (BSA) were sharply divided in their appearances (Fig. 4A). In contrast, it was observed that the signal of phosphoprotein spots after dephosphorylation with alkaline phosphatase was decreased, but non-phosphoprotein spots (BSA and phosphatase) were intensely negative (Fig. 4B). Through these results, it is attested that Calcon stain may have comparatively remarkable specificity, which shows positive and negative signals for phosphoprotein and non-phosphoproteins, respectively.
(31 – 1000 ng, 0.995), β-casein (31 – 1000 ng, 0.996), and κ-casein (62.5 – 1000 ng, 0.997) showed similar profiles with respect to the linear dynamic relationship between the Calcon and Pro-Q. It makes it possible to accurately compare the phosphoprotein expression levels and to monitor the correct phosphoprotein concentration using Calcon.

**Conclusions**

In this study, the special property of Al³⁺ permits it to act as a critical factor in specific binding with phosphorylated groups of proteins. The structure of Calcon having an O,O-dihydroxyazo group makes feasible interactions between dye and the Al³⁺-phosphoprotein complex due to the electrostatic attraction. According to the results (Supporting Information, Fig. S2), Calcon did not produce fluorescence emission for both BSA and α-casein in the absence of Al³⁺. After the addition of a certain amount of Al³⁺, however, α-casein shows a dramatic increase in the fluorescence intensity. This indicates that Al³⁺ is a phosphospecific binding element with Calcon. Further, it could be considered as to be an energy transfer channel between phosphoprotein and Calcon, leading to a complex that is responsible for the emission of fluorescence.

On the other hand, Calcon showed strong affinity to phosphoprotein-Al³⁺ by forming a ternary complex, but non-specific binding for non-phosphoproteins could be greatly reduced by the addition of SA in the destaining solution. Based on these results, the specificity of Calcon stain has been improved by significant differences between phosphoproteins representing positive signals and non-phosphoproteins with negative signals. Calcon stain may be a valid phosphoprotein staining tool that is simple, quick and suitable for the determination of phosphoprotein in SDS-PAGE, with good linearity. Calcon stain is also possible to detect phosphoproteins in 2-D SDS-PAGE. Although the sensitivity of Calcon stain is not as sensitive as Pro-Q, it should be noted that Calcon stain overcomes the problems of low specificity, time-consuming procedure, and high cost caused by other techniques (Table S1 in Supporting Information).

This detection method may monitor the phosphorylation of various proteins, such as iron-binding proteins, as well as the standard phosphoproteins used in this study, by forming a phosphate group-Al³⁺-Calcon complex. Therefore, we anticipate that the method described in this paper will be regarded as being a novel beneficial detection method in phosphoproteomics by its own merits.

**Acknowledgements**

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2016R1D1A3B03932150).

**Supporting Information**

Results of the optimal concentration determination in each step and comparisons between Calcon and Pro-Q stains. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

**References**

1. F. Marks, “Protein Phosphorylation”, Federal Republic of Germany: VCH Verlagsgesellschaft mbH and VCH Publishers, 1996, Weinheim.
2. T. Hunter, *Cell*, 2000, 100, 113.
3. G. R. Guy, R. Philip, and Y. H. Tan, *Electrophoresis*, 1994, 15, 417.
4. D. B. Kristensen, N. Kawada, K. Imamura, Y. Miyamoto, C. Tateno, S. Seki, T. Kuroki, and K. Yoshizato, *Hepatology*, 2000, 32, 268.
5. N. Kawada, D. B. Kristensen, K. Asahina, K. Nakatani, Y. Minamiyama, S. Seki, and K. Yoshizato, *J. Biol. Chem.*, 2001, 276, 25318.
6. M. R. Green, J. V. Pastewka, and A. C. Peacock, *Anal. Biochem.*, 1973, 56, 43.
7. J. Hegewauer, L. Ripley, and G. Nace, *Anal. Biochem.*, 1977, 78, 308.
8. B. Schuleenberg, R. Aggeler, J. M. Beechem, R. A. Capaldi, and W. F. Patton, *J. Biol. Chem.*, 2003, 278, 27251.
9. T. H. Steinberg, B. J. Agnew, K. R. Gee, W. Y. Leung, T. Goodman, B. Schuleenberg, J. Hendrickson, J. M. Beechem, R. P. Haugland, and W. F. Patton, *Proteomics*, 2003, 3, 1128.
10. A. Emadali, B. Muscatelli-Groux, F. Delom, S. Jenna, D. Boismenu, D. B. Sacks, P. P. Metrakos, and E. Chevet, *Mol. Cell. Proteomics*, 2006, 5, 1300.
11. J. F. Liu, Y. Cai, J. L. Wang, Q. Zhou, B. Yang, Z. Lu, L. Jiao, D. Y. Zhang, S. H. Sui, Y. Jiang, W. T. Ying, and X. H. Qian, *Electrophoresis*, 2007, 28, 4348.
12. X. Wang, S. Y. Hwang, W. T. Cong, L. T. Jin, and J. K. Choi, *Electrophoresis*, 2013, 34, 235.
13. X. Wang, S. Y. Hwang, W. T. Cong, L. T. Jin, and J. K. Choi, *Anal. Biochem.*, 2013, 435, 19.
14. S. Y. Hwang, X. Wang, W. T. Cong, L. T. Jin, and J. K. Choi, *Electrophoresis*, 2014, 35, 1089.
15. X. Wang, S. Y. Hwang, W. T. Cong, L. T. Jin, and J. K. Choi, *Electrophoresis*, 2015, 36, 2522.
16. S. Y. Hwang and J. K. Choi, *Electrophoresis*, 2017, 38, 3079.
17. G. P. Hildebrand and C. N. Reilley, *Anal. Chem.*, 1957, 29, 258.
18. Q. Zini, *Fresenius’J. Anal. Chem.*, 1992, 344, 322.
19. M. C. Posewitz and P. Tempst, *Anal. Chem.*, 1999, 71, 2883.
20. D. C. A. Neville, R. R. Townsend, C. R. Rozanas, A. S. Verkman, E. M. Price, and D. B. Gruijs, *Protein Sci.*, 1997, 6, 2436.
21. K. Sugawara, A. Yugami, and T. Kadoya, *Anal. Sci.*, 2012, 28, 251.
22. S. W. Bae, J. I. Kim, I. Choi, J. Sung, J. I. Hong, and W. S. Yeo, *Anal. Sci.*, 2017, 33, 1381.
23. R. Labugger, L. Organ, C. Collier, D. Atar, and J. E. Van Eyk, *Circulation*, 2000, 102, 1221.
24. U. K. Laemmli, *Nature*, 1970, 227, 680.
25. D. Wessel and U. I. Flügge, *Anal. Biochem.*, 1984, 138, 141.
26. X. Wang, S. Y. Hwang, W. T. Cong, L. T. Jin, and J. K. Choi, *Anal. Biochem.*, 2016, 510, 21.
27. A. E. Dahlberg, C. W. Dingman, and A. C. Peacock, *J. Mol. Biol.*, 1969, 41, 139.