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Detection and Quantification of Protein Residues in Food Grade Amino Acids and Nucleic Acids Using a Dot-Blot Fluorescent Staining Method

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Food allergies represent an important health problem in industrialized countries, such that detection and quantitative analysis of the protein considered to be the main allergen is crucial. A dot-blot fluorescent staining method for the detection and quantitative analysis of protein residues in food grade amino acids and nucleic acids is presented. This method combines fluorescence staining with dot-blotting onto PVDF membrane. Several standard proteins, such as bovine serum albumin (66 kDa), lysozyme (14 kDa), ubiquitin (8.6 kDa), bovine insulin (5.7 kDa), and oxidized insulin B chain (3.5 kDa), were detectable at 0.1 ppm using SYPRO Ruby blot stain. Twenty-five different amino acids and two different nucleic acids of food grade were analyzed using this method combined with an internal standard addition method using BSA as an internal standard. All amino acids and nucleic acids were dissolved in 3.6% aqueous HCl and dot-blotted onto PVDF membrane before a large amount of amino acids and nucleic acid were removed. Protein residues and the internal standard protein immobilized on the membrane were stained using SYPRO ruby blot stain. The internal standard in all samples was detectable at 0.1 ppm. Samples were dissolved at 120 or 70 mg/mL, according to their solubility under acidic conditions. The detection limit of protein residues per weight was 0.8−1.4 ppm in amino acids and nucleic acids; residual protein was not detected in any sample.

KEYWORDS: Amino acid; nucleic acid; monosodium glutamate; protein; allergen; quantification

INTRODUCTION

Recently, food allergy has become well recognized as an important food safety issue. Protein in eggs, peanuts, milk, hazelnut, sesame seeds, and cereals containing gluten (i.e., wheat, rye, barley, oats, spelt, kamut, or their hybridized strains) has attracted attention as causes of allergies in the EU (*1*). The main ingredient of allergens is protein. To detect allergens in food, a high sensitivity is required. At present, the enzyme-linked immunosorbent assay (ELISA), using antibodies recognizing allergens, is an extremely useful method for the detection of allergenic protein in food (*2*). In Japan, ELISAs detecting allergens in five ingredients, egg, milk, wheat, buckwheat, and peanut, are regularly used. However, very few good ELISA methods exist for other allergens.

Usually food grade amino acids and nucleic acids are produced by the fermentation method, synthetic method, or extraction method from protein hydrolysates. L-Tyr, L-Leu, and L-Cys are extracted from protein hydrolysates of plant or animal origin. In the case of fermentation, the glucose used for material is made from the starch of plant. Therefore, it is not completely denied the residual protein in amino acids and nucleic acids. These proteins may also have a risk of allergy, because allergies in food are usually caused by certain proteins.

Because food grade amino acids and nucleic acids in food are highly purified, there is little possibility that any remaining protein is present. However, to deny the present of residual proteins and to prevent protein contamination during the manufacturing process, a highly sensitive assay to detect small quantities of proteins is required. We have developed an appropriate method for the sensitive detection of protein residues in food grade amino acids and nucleic acids using dot-blot fluorescent staining. Generally, protein detection using the ELISA antigen−antibody interaction is specific and sensitive enough, although it cannot comprehensively detect all proteins. Some amino acids have low solubility when close to neutral pH, and because they cannot dissolve completely during pretreatment for the ELISA method it is difficult to perform an accurate measurement. Several methods have been developed to measure total protein in microgram quantities (*3*−*6*). Recently, a nihydrin-based assay to quantify total protein has been reported (*7*). This is a sensitive method, which can detect 2 μg of protein in a 100 μL sample; however, this sensitivity is 1 order of magnitude lower than that of the ELISA method.

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Furthermore, large quantities of amino acids and nucleic acids in a sample can affect the estimation of protein concentration using colorimetric analysis. Therefore, it is necessary to remove amino acids and nucleic acids in samples. We have established a dot-blot fluorescent staining method in which the amino acids and nucleic acids are completely dissolved and measured under acidic conditions. The sample solution is dissolved in 3.6% aqueous HCl before application onto polyvinyldiden difluoride (PVDF) membrane to immobilize residual protein in the sample onto the membrane. Immobilized residual proteins on PVDF membrane are incubated with SYPRO Ruby blot stain and detected by a laser scanner image analysis system. SYPRO Ruby blot stain is a luminescent metal chelate stain for sensitive fluorescence detection of proteins on PVDF membrane (*8*): it is more sensitive than Coomassie blue, amido black, or Indian ink (*9*). We describe a rapid and sensitive method to quantify total protein in food grade amino acids and nucleic acids.

MATERIALS AND METHODS

SYPRO Ruby protein blot stain reagent was obtained from BioRad. Milli Q water was used to wash and stain Immobilon P^{SQ} PVDF membrane (Nihon Millipore, Tokyo, Japan). Methanol and acetic acid were obtained from Junsei Chemical Co. (Tokyo, Japan), and ultrapure grade HCl was obtained from Kanto Chemical Co. (Tokyo, Japan). Standard proteins used in this study were obtained from the following sources: bovine serum albumin (BSA), ubiquitin, lysozyme, human insulin, and oxidized insulin B chain were from Sigma Chemical Co. (St. Louis, MO), and all food grade amino acids and nucleic acids were commercially available samples from Ajinomoto Co., Inc. (Tokyo, Japan). FASTKIT ELISA Kits for egg, milk, wheat, buckwheat, and peanut allergens were used (Nippon Meat Packers, Inc., Osaka, Japan).

Sample Preparation. BSA, ubiquitin, lysozyme, human insulin, and oxidized insulin B chain standards were dissolved in 3.6% aqueous HCl at concentrations of 0.1, 0.2, and 0.5 μg/mL in 1.5 mL of siliconized micro-test tubes made from polypropylene. L-Ala, L-His, L-His monohydrochloride (L-His-HCl), L-Ile, L-Leu, L-Lys monohydrochloride (L-Lys-HCl), L-Thr, L-Val, l-arginine L-glutamate (L-Arg: L-Glu), L-Cys hydrochloride monohydrate (L-Cys-HCl-H_2O), L-Ser, Gly, L-Arg, L-Pro, DL-Ala, L-theanine, and all nucleic acids were dissolved in 3.6% aqueous HCl to 120 mg/mL in 1.5 mL siliconized micro-test tubes. L-Asp, L-Cys_2, L-Glu, L-Trp, L-Gln, monosodium l-aspartate monohydrate (L-Asp-NaH_2O), monosodium L-glutamate monohydrate (L-Glu-NaH_2O, MSG), L-Phe, and L-Tyr were dissolved in 3.6% aqueous HCl to 70 mg/mL. Each sample was prepared using a final concentration of BSA between 0.1 and 0.5 μg/mL, by the dilution of 25 μg/mL protein standard solution with each amino acid or nucleic acid solution.

Dot-Blotting onto PVDF Membrane. PVDF membrane was soaked in methanol for 1−2 min for preconditioning before equilibration in 3.6% aqueous HCl for 30 min to displace the methanol. Equilibrated PVDF membrane was placed in the 96-well dot-blotting unit (AE-6190 ATTO Co., Tokyo, Japan). Next, 350 μL of 3.6% aqueous HCl was applied to each well and removed by aspiration, and then 350 μL of the sample solution was applied to each well and removed by aspiration. The membrane was then washed with 350 μL of 3.6% aqueous HCl followed with Milli Q ultrapure water. Following dot-blotting, the PVDF membrane was removed from the dot-blotting unit and air-dried for 1 h on a clean bench.

Protein Fixing and Staining. All washing and staining steps were performed at room temperature in a clean plastic container with continuous, gentle agitation on an orbital shaker (Multi Shaker MMS, Tokyo-Rikakikai Co., Ltd., Tokyo, Japan). After proteins were dot-blotted to a PVDF membrane, the membrane was floated face down in 7% acetic acid, 10% methanol and was incubated for 15 min. The membrane was washed four times in deionized water for 5 min each. Using forceps, the blot membrane was transferred to a staining dish containing SYPRO Ruby protein blot stain and was incubated for 15 min. After staining, the membrane was washed twice in deionized water for 1 min at a time to remove excess reagent.

Visualizing, Imaging, and Computer Analysis of a SYPRO Ruby Stained Blot Membrane. SYPRO Ruby stained membranes were scanned at 200 μm resolution using Typhoon Fluoroimager 8600 (Amersham Biosciences KK, Tokyo, Japan). The excitation and the emission wavelengths were 532 and 610 nm, respectively, using 610BP30 emission filter. The PTM voltage was set at 415 V. Dot-blot images were analyzed using ImageQuant software (Amersham Biosciences KK, Tokyo, Japan).

ELISA Method. The ELISA detection method for allergens in egg, milk, wheat, buckwheat, and peanut was performed according to the testing procedure for foods containing allergenic substances, specified by the Ministry of Health, Labor, and Welfare of Japan using FASTKIT ELISA Kits.

RESULTS AND DISCUSSION

Sensitivity and Linearity Analysis for Standard Proteins. In the proteome era, SYPRO Ruby has been widely used as a highly sensitive staining method for separated protein by two-dimensional gel electrophoresis. SYPRO Ruby is an organic transition metal that binds protein directly by electrostatic forces and was previously shown to detect proteins in a linear fashion and with high sensitivity. SYPRO Ruby for staining blots has only recently become commercially available for use on electro-blotted proteins. We examined the sensitivity and linear detection range of SYPRO Ruby stained proteins on PVDF membrane, which binds large and hydrophobic proteins by hydrophobic interactions, using standard proteins with differing molecular weights. To analyze residual protein within an amino acid sample at high sensitivity, using weight ratio, the protein must be in a soluble state. Generally, amino acids are soluble under acidic conditions; therefore, we dissolved our proteins in 3.6% HCl solution. For accuracy, it is important to avoid adsorption of proteins to containers during sample preparation as much as possible. The dilution step was reduced as much as possible using siliconized micro-test tubes and tips. The standard protein stock solutions were prepared at 25 μg/mL in 3.6% aqueous HCl. Each standard protein stock solution was then diluted to 0.5, 0.2, and 0.1 μg/mL with 3.6% HCl. Next, 350 μL of each standard protein solution was applied, by dot-blotting, to washed and preconditioned PVDF membrane. Samples were applied in quadruplicate on each membrane. To obtain high reproducibility, it is important to dry the PVDF membrane before staining. Between 35 and 175 ng of protein was present within the spots on the PVDF membrane. Proteins on the membrane were stained using SYPRO Ruby protein blot stain and visualized by a laser scanner image analysis system. Following visualization, the obtained images were analyzed using ImageQuant software (Amersham Biosciences KK, Tokyo, Japan). The results of the standard proteins, BSA (66 kDa), lysozyme (14 kDa), ubiquitin (8.6 kDa), bovine insulin (5.7 kDa), and oxidized insulin B chain (3.5 kDa), are shown in **Figure 1**. In the case of all of the standard proteins, 35 ng/well (0.1 ppm) of protein was detectable on the dot-blotted membrane using SYPRO Ruby blot stain. These results indicate the linear range for protein detection range was 35−175ng/well. In the quadruplicate spots, CV values are 2−15% on same membrane. The slope of the calibration curve changes with differences in staining and imaging processing between different membranes.

Sensitivity and Linearity Analysis of Protein in Food Grade Amino Acids and Nucleic Acids. Because the amino acids and nucleic acids of food grade are purified, there is little possibility that any contaminating or remaining protein is present. The presence of amino acids, however, obscures the detection of contaminating protein; using the BCA or Bradford method, it is difficult to detect total protein below 10 ppm. To

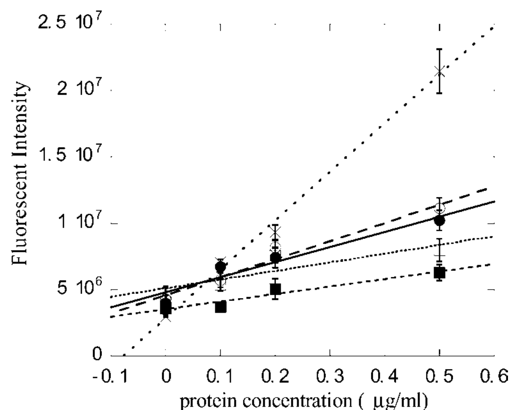

Figure 1. Detection sensitivity and linear range of the dot-blot fluorescent staining method: ■, oxidized insulin B chain; □, insulin; ●, bovine serum albumin; ○, ubiquitin; ×, hen egg lysozyme.

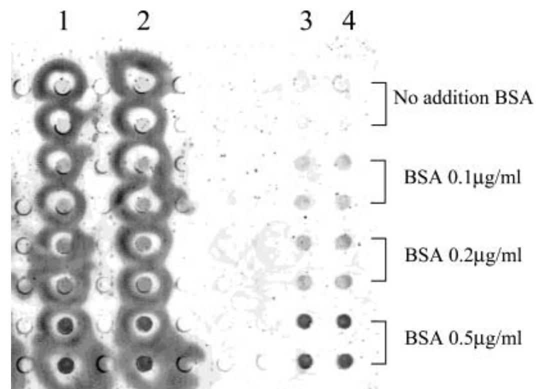

Figure 2. Dot-blot fluorescent stained PVDF membrane using the internal standard addition method: lanes 1 and 2, Leu with 0.1 μg–0.5μg/mL BSA as internal standard; lanes 3 and 4, BSA.

Table 1. Solubility of Amino Acids in Water

amino acid	solubility g/dL		
	H_2O, 0 °C[a]	H_2O, 25 °C[a]	3.6% HCl, room temp
L-Asp		0.45 (20 °C)	14
L-Cys_2		0.0112	8
L-Ile	3.79	4.12	18
L-Leu	2.27	2.426	13
L-Phe	1.98	2.96	13
L-Tyr	0.02	0.045	8
L-Val	8.34	8.85	21

[a] *The Merck Index*, 12th ed.; Budavari, S., Ed.; Merck & Co., Inc.: Rahway, NJ, 1996.

detect protein with higher sensitivity, it is necessary to measure the sample when it has been dissolved as much as possible. The solubility of amino acids is higher under acidic conditions than it is under neutral conditions. In particular, the solubility of L-Tyr or L-Cys_2 was remarkably improved under acidic conditions. The approximate solubility of amino acids used in this study in 3.6% HCl solution is summarized in **Table 1**. In acid solution, the solubility of L-Tyr and L-Cys_2 increased to 8 g/dL from 0.04 and 0.01 g/dL, respectively. From this, a 200–800 times improvement in the sensitivity of total protein per weight can be expected. L-Asp, L-Cys_2, L-Glu, L-Trp, L-Gln, L-Asp-NaH_2O, L-Glu-NaH_2O, L-Phe, and L-Tyr, amino acids with comparatively low solubility, were dissolved at 70 mg/mL. Other amino acids were prepared at concentrations of 120 mg/mL in 3.6% aqueous HCl. For consistency, the nucleic acids were also dissolved in the same acidic solution even though they dissolved well in water (GMP approximately 25 g/dL H_2O; IMP approximately 13 g/dL H_2O).

Analysis of total protein in amino acid and nucleic acid samples was performed via the standard addition method using BSA as the protein standard. For an improvement in analysis accuracy, BSA was applied on all PVDF membranes as an external standard (**Figure 2**).

For the external standard protein, the fluorescence of BSA was dependent on concentration. As can be seen in **Figure 2**, 0.1 μg/mL (0.1 ppm) spots are clearly detected. The fluorescence of some amino acid samples in which BSA has been added were observed as a concentric circle around the main spot of protein. These are considered to be amino acids that have soaked into the PVDF membrane at the time of application and have not been removed by washing. Existence of a concentric circle but no fluorescence within that circle indicates that the amino acid on the central spot was fully washed. The fluorescent

Table 2. Protein Residues in Food Grade Amino Acids and Nucleic Acids Using the Dot-Blot Fluorescent Staining Method

sample	sample concn	BSA detection limit	detection limit per sample weight	results
L-Ala L-His L-His-HCl L-Ile L-Leu L-Lys-HCl L-Thr L-Val L-Arg:L-Glu L-Cys-HCl-H_2O L-Ser Gly L-Arg L-Pro DL-Ala L-theanine	120 mg/mL	0.1 mg/mL (0.1 ppm)	0.1 mg of BSA/ 120 mg of osample = 0.8 ppm	blow detection limit
L-Asp (L-Cys)$_2$ L-Glu L-Trp L-Gln L-Asp-NaH_2O L-Glu-NaH_2O L-Phe L-Tyr	70 mg/mL	0.1 mg/mL (0.1 ppm)	0.1 mg of BSA/ 70 mg of sample = 1/4 ppm	blow detection limit
GMP IMP	120 mg/mL	0.1 mg/mL (0.1 ppm)	0.1 mg of BSA/ 120 mg of sample = 0.8 ppm	blow detection limit

intensity within spots of amino acids with added BSA was of the same intensity as the external standards: spots containing amino acid sample with 0.1 μg/mL BSA fluoresced at 1 ppm of protein in an amino acid weight ratio. Not only does this method detect proteins with high sensitivity, it also detects a broad molecular weight range. The results from food grade amino acids and nucleic acids measured using this method are summarized in **Table 2**. L-Ala, L-His, L-His-HCl, L-Ile, L-Leu, L-Lys-HCl, L-Thr, L-Val, L-Arg:L-Glu, L-Cys-HCl-H_2O, L-Ser, Gly, L-Arg, L-Pro, DL-Ala, l-theanine, and all nucleic acids were dissolved in 3.6% aqueous HCl and concentrated to 120 mg/mL in 1.5 mL siliconized micro-test tubes. BSA standard solution (25 μg/mL) was prepared by a 40 times dilution of 1 mg/mL BSA solution with 120 mg/mL of amino acid solution.

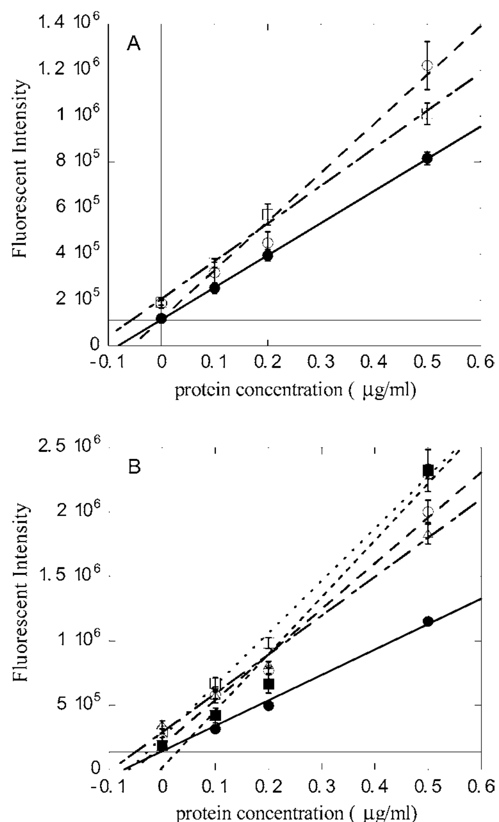

Figure 3. Detection of protein residue in food grade amino acids using the dot-blot fluorescent staining method with the internal standard addition method: **(A)** ●, bovine serum albumin; □, monosodium L-glutamate monohydrate; ○, L-theanine; **(B)** ●, bovine serum albumin; ○, Gly; ■, L-Arg; △, L-Tyr; □, L-Pro.

This was further diluted with the same amino acid solution 50 times, 125 times, and 250 times. Next, 0.1, 0.2, and 0.5 μg of BSA/120 mg of amino acid/1 mL of 3.6% HCl solutions were prepared for the standard addition method. The protein concentrations of these samples were set to 0.1, 0.2, and 0.5 ppm per volume and 0.8, 1.6, and 4.2 ppm per amino acid weight. Several amino acids (L-Asp, L-Cys_2, L-Glu, L-Trp, L-Gln, L-Asp-NaH_2O, L-Glu-NaH_2O, L-Phe, and L-Tyr) were dissolved at a concentration of 70 mg/mL, and the protein concentrations were then set to 1.4, 2.9, and 7.1 ppm per amino acid weight. The minimum protein concentration was detected in all samples.

The fluorescence intensity of these spots was integrated using ImageQuant software (Amersham Biosciences KK, Tokyo, Japan). Graphs showing the average value of four spots per sample are shown in **Figure 3**. Both BSA and BSA-added amino acid sample showed a linear detection range; the CV value was between 2% and 13%. Intersection with the *Y*-axis of the standard curve for BSA, the external standard, was set as zero, that is, an intersection with the *X*-axis. To improve accuracy in the amino acid samples, the intersection of the *X*-axis in the standard curve of BSA-added samples was calculated as the protein concentration for each amino acid. For all samples, the intersection with the *X*-axis is between 0 and −0.1 ppm (**Figure 3**). The results shown in **Table 2** are below the detection limit of 1 or 1.4 ppm, depending on the sample concentration.

As a comparison, the ELISA detection method for egg, milk, wheat, buckwheat, and peanut allergens was performed according to the testing procedure for foods containing allergenic substances, specified by the Ministry of Health, Labor, and Welfare of Japan. In the case of all amino acids and nucleic acids, all five ingredients were not detected by using the ELISA methods. In this method, protein derived from specified ingredients in certain foods was detected at 1 ppm below the detection limit. In the case of easy soluble amino acids in water, the detection limit of the ELISA method is the same as that of our method. In the preparation procedure for ELISA, 2 g of sample was resuspended to 38 mL using extraction buffer solution and was extracted at near neutrality (pH 6.0−8.0). Some amino acids with low solubility, especially L-Cys_2 and L-Tyr, were not dissolved under these conditions (**Table 1**). Acidic conditions are not suitable for the ELISA method because antibody cannot bind to antigen. In contrast, our method can be performed under acidic conditions to dissolve every amino acid and nucleic acid. Therefore, quantification and detection of residual protein in amino acids and nucleic acids can be performed with higher accuracy and sensitivity. In addition, in our procedure the amount of total protein can be measured without the need to prepare antibodies for each specified ingredient, and peptides as small as 3.5 kDa can be detected. The analytical time, cost, and technical expertise of our method are almost the same as those of the ELISA method.

Our method is able to improve the sensitivity in the case of easy soluble amino acids and nucleic acids because the its detection limit depends on the sample solubility. The solubility of Pro is 127 g/dL under acidic conditions, and the solubility of GMP is 25 g/dL. So, the calculated best detection limits of Pro and GMP are 0.08 and 0.4 ppm, respectively.

In this paper, we developed a total protein detection method for amino acids and nucleic acids. Certain proteins primarily cause allergies in food. Unfortunately, the identification and assays for allergenic proteins are not available; therefore, the highly sensitive total protein measurement is very useful for the primary assay of allergenic potential.

We think our method can be applied to other purified food ingredients and additives, for example, amino acids derivatives, vitamins, oligosaccharides, sugar, sugar derivatives, dextrin, and cyclodextrin. In addition, our total protein assay can be applied to detect the residual proteins from the ethics of genetically modified organisms (GMO). It is very important to ensure no contamination with GMO protein to assess the product safety.

In addition, total protein assays are needed beyond monitoring food grade amino acids. For example, proteins from natural rubber latex products can cause severe allergies. Gloves made from rubber latex are used for medical and food handling; therefore, humans and food are exposed to these allergens.

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LITERATURE CITED

(1) Official Journal of the European Union L 308, 25, 11, 2003; p 15.

(2) Koppelman, S. J.; Knulst, A. C.; Koers, W. J.; Penninks, A. H.; Peppelman, H.; Vlooswijk, R.; Pigmans, I.; van Duijn, G.; Hessing, M. Comparison of different immunochemical methods for the detection and quantification of hazelnut proteins in food products. *J. Immunol. Methods* **1999**, *229*, 107−20.

(3) Lowry, O. H.; Rosenborugh, N. J.; Farr, A. L. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

(4) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(5) Alam, A. A model for formulation of protein assay. *Anal. Biochem.* **1992**, *203*, 121−126.

(6) Gotham, S. M.; Fryer, P. J.; Paterson, W. R. The measurement of insoluble protein using a modified Bradford assay. *Anal. Biochem.* **1988**, *173*, 3553−358.

(7) Starcher, B. A ninhydrin-based assay to quantitate the total protein content of tissue samples. *Anal. Biochem*. **2001**, *292*, 125−129.

(8) Berggren, K.; Steinberg, T. H.; Lauber, W.; Carroll, J. A.; Lopez, M.; Chernokalskaya, E.; Zieske, L.; Diwu, Z.; Haugland, R. P.; Patton, W. A luminescent ruthenium complex for ultrasensitive detection of proteins immobilized on membrane supports. *Anal. Biochem*. **1999**, *276*, 129−143.

(9) Kemper, C.; Berggren, K.; Diwu, Z.; Patton, W. F. An improved, luminescent europium-based attain for detection of electroblotted proteins on nitrocellulose or poly(vinylidene difluoride) membranes. *Electrophoresis* **2001**, *22*, 881−889.

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