Supporting Information for:

Sensitive Luminometric Method for Protein Quantification in Bacterial Cell Lysate Based on Particle Adsorption and Dissociation of Chelated Europium

Sari Pihlasalo, Antti Kulmala, Anita Rozwandowicz-Jansen, Pekka Hänninen, and Harri Härmä*

Analytical Chemistry

Labeling of Amino-modified Polystyrene Particles with Eu^{3+} Chelates

Amino-modified polystyrene particles (Spherotech Inc., Libertyville, IL), 240 nm in diameter, were labeled with 7-dentate Eu^{3+} chelate, 2,2',2'',2'''-{[4-[(4-isothiocyanatophenyl)ethynyl]pyridine-2,6-diyl]bis(methylenenitrilo)}tetrakis(acetato) europium, and 9-dentate Eu^{3+} chelate, {2,2',2'',2'''-{[4'-(4'''-isothiocyanatophenyl)-2,2':6,6''-terpyridine-6,6''-diyl]bis(methylenenitrilo)}tetrakis (acetato)} europium, at varying chelate concentrations. The chelates were from the Laboratory of Biophysics (Turku, Finland) and were synthesized and characterized according to the literature.[1,2] Before labeling, the particles were washed by centrifugation (19,000g for 5 min) several times. The 7-dentate (1.0-21 nmol) and 9-dentate (0.39-12 nmol) Eu^{3+} chelates were added to 125 µL of 30 mM carbonate buffer, pH 9.9, (sodium hydroxide from FF-Chemicals, Yli-Ii, Finland) containing 46 fmol of particles. After overnight incubation, the particles were washed in water by centrifugation (19,000g for 5 min) several times. The number of Eu^{3+} chelates per particle was determined with DELFIA enhancement solution (Wallac, Perkin-Elmer Life and Analytical Sciences, Turku, Finland), as recommended by the manufacturer, except that the europium ions were first dissociated from the chelates in 1 M hydrochloric acid (concentrated hydrochloric acid from Sigma-Aldrich Co., St. Louis, MO) before mixing and enhancing with DELFIA.

Zeta Potential Measurements for Unlabeled Amino-modified Polystyrene Particles and Surface-labeled with 9-dentate Eu^{3+} Chelate

Zeta potentials were measured with a Zetasizer Nano Series Nano ZS instrument (Malvern Instruments Ltd, Malvern, U. K.). The zeta potential of unlabeled amino-modified polystyrene particles (44 pM) was measured in water, 3 mM acetate (FF-Chemicals, Yli-Ii, Finland) buffer, pH 5.0, and a universal buffer containing 0.5 mM sodium tetraborate (Sigma-Aldrich Co., St. Louis, MO), citric acid (FF-Chemicals, Yli-Ii, Finland), tris(hydroxymethyl)aminomethane (Calbiochem-Novabiochem Co., La Jolla, CA), potassium dihydrogen phosphate (Merck KGaA, Darmstadt, Germany), and potassium chloride (J. T. Baker, Deventer, Holland) with a pH varying from 2.5 to 11.5. The zeta potential of particles that were surface-labeled with 9-dentate Eu^{3+} chelate (75,000 chelates per particle, 44 pM) was measured in water.

Verification of Dissociation of Lanthanide Ion from Eu^{3+} Chelate

Dissociation of the Eu^{3+} ion from the Eu^{3+} chelate was verified with a test in which the particles were separated after the dissociation of the Eu^{3+} ion from the chelate at a low pH. The europium particles (labeled with 9-dentate chelate, 12,000 chelates per particle, 1.1 fmol) were incubated in 160 µL of water or 60 mM glycine buffer, pH 2.0. After 30 min, the particles were removed by centrifugation (19,000g for 5 min). The Eu^{3+} concentration of the supernatant was determined with a DELFIA enhancement solution.

Optimization of Eu^{3+} Chelate Type, Number of Particles, and Incubation Temperature in Assay

Eu^{3+} chelate type: Two Eu^{3+} chelates, 7-dentate and 9-dentate Eu^{3+} chelates, were compared. Different concentrations of BSA and europium particles labeled with 9-dentate (13,000 chelates per particle, 18 amol) or 7-dentate chelate (27,000 chelates per particle, 18 amol) were mixed in 75 µL of 3 mM acetate buffer, pH 5.0. After mixing, 20 µL of a hydrochloric acid solution with varying concentrations was added to obtain different final pH values.

Number of particles: The assay for protein quantification was optimized for the particle concentration by running the BSA calibration curve with a varying number of particles (labeled with 9-dentate chelate, 52,000 chelates per particle) in 75 µL of 3 mM acetate buffer, pH 5.0. 20 µL of a dissociation buffer of 0.5 M glycine, pH 2.0 was added to obtain the final pH.

Incubation temperature: Different concentrations of BSA and europium particles (labeled with 9-dentate chelate, 1,800 chelates per particle, 41 amol) were mixed in 170 µL of 3 mM acetate buffer, pH 5.0 and incubated at different temperatures (room temperature, 37 °C, 55 °C, and 100 °C) for 35 min. After mixing, 75 µL of the solution was transferred into microtiter wells and 20 µL of a hydrochloric acid solution was added to obtain the final pH of 2.0.

Bacterial Culture, Lysis, and Production and Purification of Antibody Fragment

An antibody fragment (Fab) was produced in transfected *Escherichia coli* XL-1. The pre-culture was obtained by inoculating 20 mL super broth (SB) (0.1 g/L ampicillin and 5 g/L glucose as supplements) with single colony of freshly streaked LA plate and grown at 30 °C with 300 rpm shaking overnight. For the main culture, 250 mL SB (0.1 g/L ampicillin and 10 g/L glucose as supplements) was inoculated with the preculture to optical density of 0.05 at 600 nm and grown (37 °C, 300 rpm) to optical density of 0.5 at 600 nm. The culture medium was changed into glucose free and the culture was induced with 0.25 mM isopropyl *β*-D-1-thiogalactopyranoside and grown (26 °C, 250 rpm) overnight.

In total four samples were prepared from the culture, samples 1-3 in phosphate buffered saline (PBS) (Lonza, Basel, Switzerland) and sample 4 in 50 mM Tris, pH 7.5, containing 150 mM NaCl (TBS). The bacteria were harvested in PBS buffer by centrifugation (10 000g, 15 min) (sample 1). The concentration of the bacteria 7×10^9/mL was approximated from the optical density reading at 600 nm. Part of the bacteria was further sonicated using a Labsonic U tip sonicator (B. Braun Biotech International GmbH, Melsungen, Germany) on ice (sample 2). Part of the sonicated bacteria was centrifuged (10 000g, 20 min) and the pellet was discarded to obtain sample 3. To prepare sample 4, the bacteria were originally harvested in TBS and the steps similar to the samples 1-3 were carried out. Fab was purified from the supernatant with the use of 1 mL HiTrap™ Protein G column (GE Healthcare, Uppsala, Sweden), 20 mM sodium phosphate, pH 7.0 as a binding buffer, and 0.1 M glycine, pH 2.7 as an elution buffer. The 500 µL fractions were collected and the pH was neutralized with Tris buffer. The fractions containing purified Fab were observed from absorbance reading at 280 nm and were combined to obtain sample 4.

Quantification of Produced and Purified Antibody Fragment and Protein in Bacterial Lysates

The protein concentration in prepared four samples was measured with four different commercial or literature methods (BCA, NanoOrange, UV280, and corrected UV280) and compared to the developed dissociation method. The total protein was quantified with BCA (Thermo Fisher Scientific (Pierce) Inc., Rockford, IL) and NanoOrange (Life Technologies Co., Carlsbad, CA) methods according to the kit instructions provided by the manufacturer. Quantification from the absorbance at 280 nm (UV280) was performed in PBS using disposable UV micro cuvettes. In this method, the extinction coefficient of protein or protein mixtures was assumed as 1 L g^{-1} cm^{-1}. Quantification from the absorbance at 280 nm corrected with the absorbance at 260 nm (corrected UV280) was performed similarly to UV280. In this method,[3] the interference of the nucleic acid in the UV280 reading was eliminated in the calculation the concentration of total protein by using an empirical formula,

$$c = \frac{a \times A(280\ \text{nm}) - b \times A(260\ \text{nm})}{l}$$

in which c = concentration of protein, l = optical path length, A = absorbance, and constants a = 1.55 g cm L^{-1} and b = 0.76 g cm L^{-1}. Quantification with the developed dissociation method was carried as described in Experimental Section. In each method, the appropriate dilutions were prepared from the samples and the BSA standard (no standard in UV280 and corrected UV280) to the assay buffer to cover the dynamic range of the method.

Supporting Results

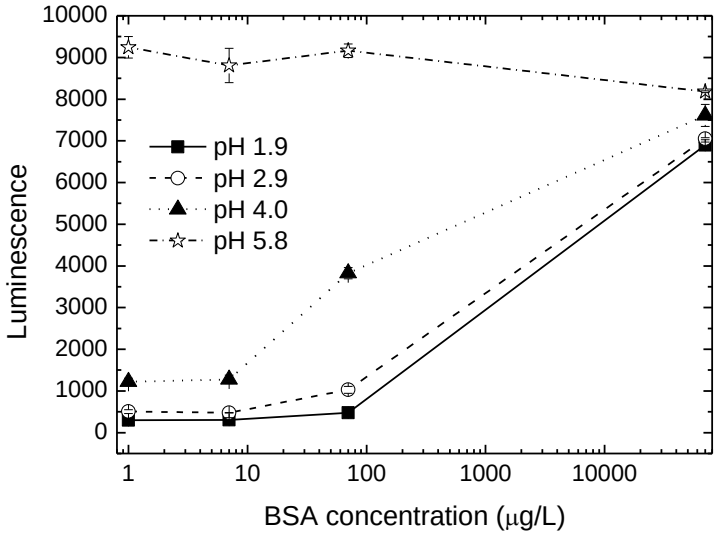

Figure S1. Effect of pH on the dissociation of the Eu^{3+} ion from the 9-dentate Eu^{3+} chelate at different concentrations of bovine serum albumin (BSA). The pH was changed with a hydrochloric acid solution after the incubation of BSA and particles in water.

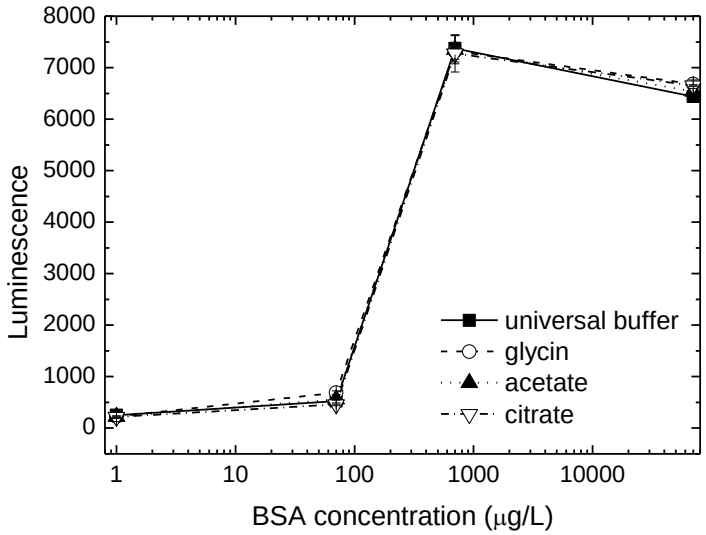

Figure S2. Effect of the protein adsorption buffer component on the assay. BSA calibration curves were run by adsorbing the protein to the Eu^{3+} particles containing the 9-dentate chelate in different buffers at a 3.0 mM concentration (0.50 mM for each universal buffer component) at pH 5.0.

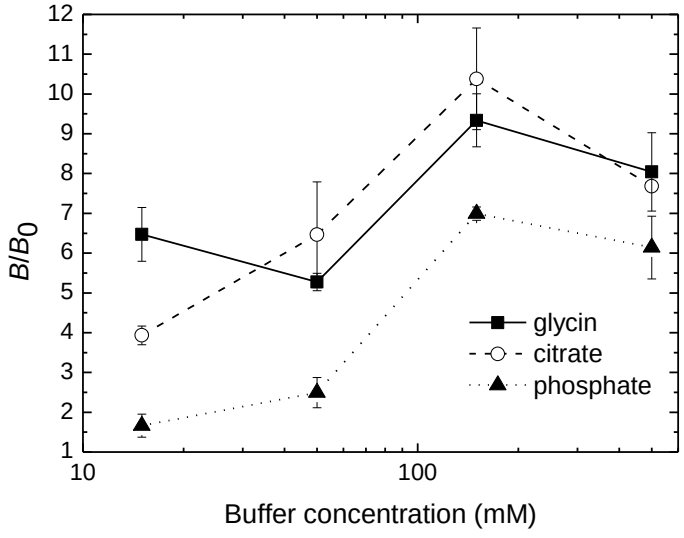

Figure S3. Effect of the dissociation buffer components in varying concentrations and at pH 2.0 on the ratio between the luminescence signals B/B_0 at 6.5 and 0 mg/L bovine serum albumin (BSA). 6.5 and 0 mg/L BSA was incubated with particles containing the 9-dentate Eu^{3+} chelate in a 3.0 mM acetate buffer pH 5.0, after which one of the dissociation buffers was added.

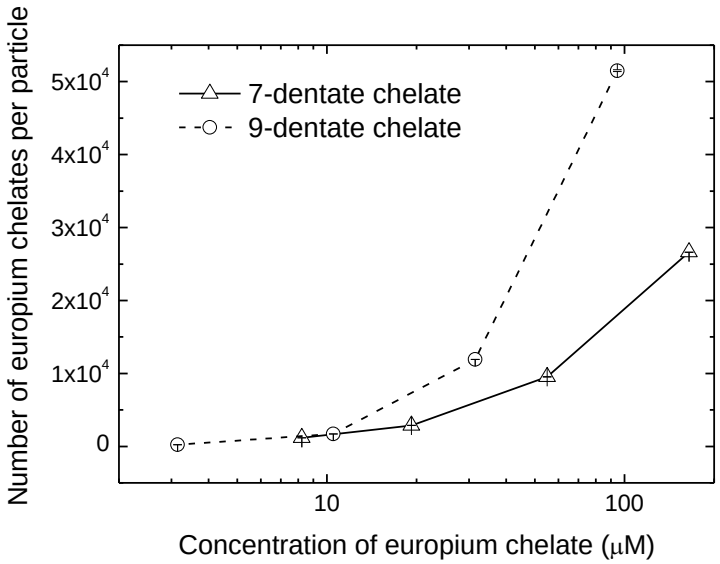

Figure S4. Relation between the concentration of Eu^{3+} chelate used in the labeling of amino-modified polystyrene particles, 240 nm in diameter, and the number of Eu^{3+} chelates bound per particle for two chelate types: 7-dentate and 9-dentate Eu^{3+} chelates.

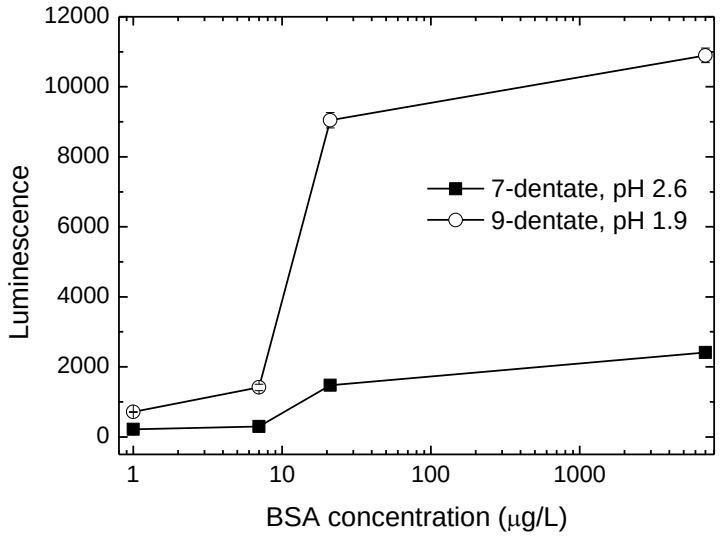

Figure S5. Comparison of particles labeled with 7-dentate or 9-dentate Eu^{3+} chelates for the quantification of proteins. BSA was adsorbed in a 3 mM acetate buffer at pH 5.0, and the final pH was varied by adding different concentrations of a hydrochloric acid solution.

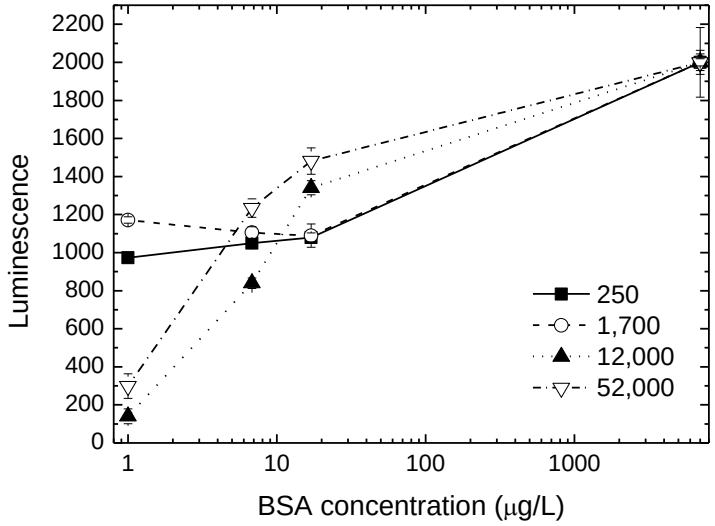

Figure S6. Effect of the particle labeling degree on the assay. The calibration curve for bovine serum albumin (BSA) measured using particles labeled with 9-dentate Eu^{3+} chelate at varying numbers per particle for a fixed luminescence signal (measured values normalized to exactly 2,000 at 6300 μg/L BSA concentration) at a high BSA concentration level.

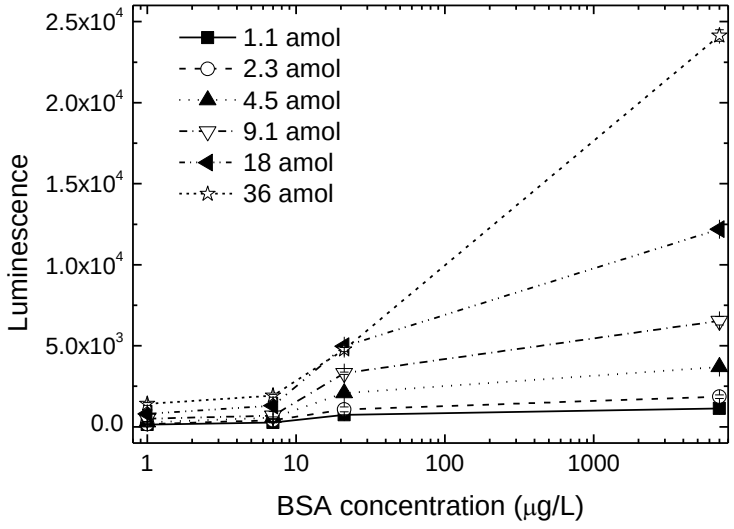

Figure S7. Effect of particle number (1.1-36 amol) on the quantification of bovine serum albumin (BSA). The BSA calibration curves were measured with varying number of particles per well.

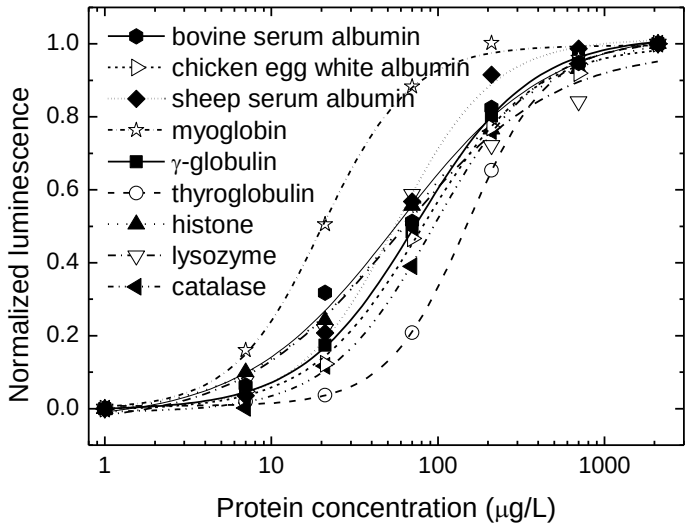

Figure S8. Response curves of different proteins measured with the developed method presented as a normalized background reduced luminescence signal, and as a function of protein concentration. The data were fitted to the modified Hill function.

Table S1. Literature values of isoelectric points for each tested protein.

Protein	Isoelectric point
Bovine serum albumin	4.90[4]
Chicken serum albumin	4.7[5]
Sheep serum albumin	5.80[6-8]
Myoglobin	7.33/7.45[5]
γ-globulin	6.4-8.8[9]
Thyroglobulin (hog)	4.5[10]
Histone	10.8[11]
Lysozyme	11.35[12]
Catalase	5.4[13]

Discussion for Quantification of Total Protein for Antibody Fragment and Bacterial Lysate Samples

The protein concentration in four real samples was measured with four different commercial or literature methods (BCA, NanoOrange, UV280, and corrected UV280) and compared to the developed dissociation method. The determined concentration of protein in purified Fab sample 4 was quite similar for all five methods. Furthermore, the results are adequately similar for supernatant sample 3 measured with all methods except UV280. The concentration for UV280 is

clearly higher than for other methods. We consider that it results from the interfering nucleic acid in the sample. Even higher discrepancies exist for *E. coli* and lysed *E. coli* samples 1 and 2, which contain the bacterial membranes. The difference between the protein concentration for lysed *E. coli* and its supernatant samples 2 and 3 quantified with BCA and NanoOrange methods might originate from the ability of these methods to detect the protein still attached to the bacterial membrane in addition to the free soluble protein. This explanation cannot be applied to the UV methods, as the measured protein concentrations are too high compared to the total mass of *E. coli*. According to the UV280 method one *E. coli* contains 10 pg protein and according to the corrected UV280 7 pg protein calculated from the result for sample 2, while the weight of *E. coli* is approximately 1 pg.[14] Thus, the results are incorrect most probably due to the scattered light produced by the bacterial membranes. Moreover, the concentrations measured with the same method cannot be similar for samples 1 and 2, if the reagents do not lyse the bacteria in the sample 1.

With the developed dissociation method, nearly identical concentration of protein was measured for samples 2 and 3, which suggests that the method quantifies only the protein that is detached from the bacteria. Probably, the nanoparticles in the developed method cannot approach the protein attached to the membrane, as the particles are larger compared to the single dye molecules in BCA and NanoOrange methods. Thus, the results suggest that the developed method quantifies protein in the presence of bacterial lysate, while the existing methods, BCA, NanoOrange, and UV absorbance methods fail to measure the protein concentration without purification. Moreover, the determined concentration of protein was lower for the intact *E. coli* sample 1 compared to the lysed samples 2 and 3, which supports our view. The results suggest that the dissociation method can be used for the sensitive quantification of real samples. Sensitive methods are needed, as the concentration of the purified Fab (sample 4) was quite low. The protein concentration is close to the detection limit of the BCA and UV methods and the quantification would be unreliable, if the concentration was still slightly lower. This test suggests that more sensitive methods are needed. If the concentration was lower, the protein could not be quantified with these methods. Furthermore, the BCA and UV methods require high sample volume. Instead, the considerably diluted sample is sufficient for the developed dissociation method.

References

1. Hakala, H.; Liitti, P.; Puukka, K; Peuralahti, J.; Loman, K.; Karvinen, J.; Ollikka, P.; Ylikoski, A.; Mukkala, V.-M.; Hovinen, J. *Inorg. Chem. Commun.* **2002**, *5*, 1059−1062.

2. Takalo, H.; Mukkala, V.-M.; Mikola, H.; Liitti, P.; Hemmilä, I. *Bioconjugate Chem.* **1994**, *5*, 278−282.

3. Stoscheck, C. M. *Method. Enzymol.* **1990**, *182*, 50–68.

4. Conway-Jacobs, A.; Lewin, L. M. *Anal. Biochem.* **1971**, *43*, 394–400.

5. Righetti, P. G.; Caravaggio, T. *J. Chromatogr.* **1976**, *127*, 1–28.

6. Protein Identification and Analysis Tools on the ExPASy Server, web.expasy.org/compute_pi/

7. Gasteiger, E.; Hoogland, C.; Gattiker, A.; Duvaud, S.; Wilkins, M. R.; Appel, R. D.; Bairoch, A. In *The Proteomics Protocols Handbook*, Walker, J. M., Ed., Humana Press, 2005, pp. 571–607.

8. www.ncbi.nlm.nih.gov/pubmed/

9. Condie, R. M. Pure intravenous human and animal gamma globulins. U.S. Patent 4,296,027, October 21, 1981.

10. Ui, N. *Biochim. Biophys. Acta* **1972**, *257*, 350–364.

11. Hayashida, O.; Uchiyama, M. *Tetrahedron Lett.* **2006**, *47*, 4091–4094.

12. Wetter, L. R.; Deutsch, H. F. *J. Biol. Chem.* **1951**, *192*, 237–242.

13. Gorbunoff, M. J. *Anal. Biochem.* **1984**, *136*, 425–432.

14. Davis, B. D.; Dulbecco, R.; Eisen, H. N.; Ginsberg, H. S. *Microbiology*, 2nd ed.; Harper & Row, Hagerstown, Maryland, 1973.