

Quantitation of DNA from Flow Cytometry Analysis Using Fluorescent Probe

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A reproducible method to quantify DNA in DNA and protein solutions at ppb levels using flow cytometry analysis is reported herein. After DNA and protein solutions were dyed with fluorescent probe (PicoGreen™) intercalating, flow cytometric scattergrams of DNA at fluorescent intensities of 525 nm and 575 nm showed a higher intensity of emission at 525 nm than those of proteins. DNA quantitation was performed using calf thymus DNA with two standard curves. The high standard curve showed a semi-logarithmic plot from 1 to 50 ppb with a correlation coefficient of 0.993, whereas the low standard curve described the second-power equation from 0.1 to 5 ppb DNA with a correlation coefficient of 0.998. A flow cytometry analysis of the DNA concentration was powerful for estimating the DNA concentration in a protein solution containing salts, such as saline, because the present flow cytometry analysis showed less than a 37% decline when the DNA solution contained 0.15 mol/L NaCl. The DNA content in albumin, cellulase, γ-globulin, and pepsin was estimated to be 0.078 ± 0.006, 0.26 ± 0.04, 3.0 ± 0.4, and 1.2 ± 0.1 μg-DNA/g-protein ($n = 4$), respectively, based on flow cytometry analysis of the protein solutions.

A concern over DNA hazards was considered in the mid-1950s, because there was a growing concern that the products might contain oncogenic sequences in viral vaccines.[1] The quantitation of DNA is therefore important in therapeutic biopharmaceuticals and in vivo diagnostics made from recombinant DNA and monoclonal antibodies, in order to prevent potential safety hazards.[2–8]

Kung et al. reported a rapid and reproducible method to quantify DNA at pg/mL levels.[6] Two high-affinity DNA-binding proteins (single-stranded DNA-binding protein (SSB) and monoclonal anti-DNA antibodies) were used to construct a sandwich assay. A semiconductor sensor that detected DNA with a light-addressable potentiometric sensor was used for the quantitation of DNA. This assay could detect 2 pg/mL of DNA with a quantitation coefficient of variation of less than 10% in the range of 2 to 200 pg/mL.[6] However, currently, this DNA quantitation method is not typically used because semiconductor sensors and DNA quantitation kits are extremely expensive.

Bolger et al. developed a simple and rapid assay to quantify the total DNA in protein solutions over a wide range of conditions.[1] Their total DNA quantitation assay was a homogeneous fluorescence-based DNA detection system. The technique relied on an increase in the fluorescence of a dye molecule when it intercalated into double-stranded DNA. The assay was found to be linear from 98 to 200 ng/mL of DNA in buffers containing no proteins, and was found to have a detection limit of 1 ng/mL in a typical protein solution.

This method of fluorescence-based DNA detection was also used to quantify the DNA concentration in a protein solution using fluorescence spectroscopy in our previous studies.[4,5] The fluorescence peak of DNA at 530 nm appeared, but was sometimes hidden on the shoulder of an emission spectra for a protein solution. This is because the emission spectroscopy of a protein solution containing trace amounts of DNA and a fluorescence probe gave a high emission intensity from protein that interfered with the emission spectra of DNA. The quantification of DNA in the protein solution was found to be extremely difficult when the concentration of DNA in this solution was less than 10 ppb using this method.

The particle size for a cell culture medium spiked with serum was detected in forward and side scattering flow cytometry measurements in our laboratory when the size of animal cells, such as hepatocytes and lymphocytes, was analyzed using flow cytometry.[9,10] This result prompted us to propose that DNA intercalated with a fluorescence probe in the protein solution might be precisely detected using flow cytometry analysis.

Herein, the quantitation of DNA analyzed by flow cytometry is reported. The DNA concentration in several protein solutions is also reported.

Experimental

Materials. γ-Globulin (Bovine, Cohn fractions II, III, Lot 58H7610), albumin (Bovine, fatty acid free, Lot 072K0991) were purchased from Sigma Chemical Co. Cellulase (from Aspergillus niger, C0057, Lot FGL01) was purchased from Tokyo Kasei Kogyo Co., Ltd. Pepsin (from porcine stomach mucosa, 162-18721, Lot WAG7073) and trypsin (from bovine pancreas, 548-00212, Lot NUG2121) were purchased from Wako Pure Chemical Industries, Ltd. Calf thymus DNA was purchased from the Sigma Chemical Co. PicoGreen™ ds DNA quantitation reagent (P-7581) was purchased from Molecular Probes, Inc. All other chemicals used were of reagent grade, and were used without further purification. Ultrapure water was used throughout the experiments.

Preparation of DNA Solution. DNA was dissolved in 5 mmol/L tris(hydroxymethyl)aminomethane·HCl/0.5 mmol/L

Published on the web November 11, 2004; DOI 10.1246/bcsj.77.2021

ethylenediaminetetraacetic acid (EDTA) buffer, adjusted to a pH of 8.0 by adding 0.1 mol/L acetic acid. NaCl was added into the DNA solution (0.1–50 ppb), and DNA solutions containing either 0 or 0.15 mol/L NaCl were used. C_{DNA} indicates the concentration of DNA in the DNA solution.

Preparation of Protein Solution. Albumin, cellulase, γ-globulin, pepsin, and trypsin were dissolved in 5 mmol/L tris(hydroxymethyl)aminomethane·HCl/0.5 mmol/L ethylenediaminetetraacetic acid (EDTA) buffer, and adjusted to a pH of 8.0 by adding 0.1 mol/L acetic acid. NaCl was added into the protein solution; protein solutions containing either 0 or 0.15 mol/L NaCl were used.

Flow Cytometry Analysis of DNA and Protein Solutions. Flow cytometry analysis of the DNA and protein solution was performed on an EPICS XL ADC (Beckman Coulter, Inc.); the data were collected using logarithmic amplification. For each sample, 0.5 mL of DNA or a protein solution containing 2.5 μL of PicoGreen™ was counted in the forward scattering, side scattering, fluorescent intensity at 525 nm, and fluorescent intensity at 575 nm using an Ar laser (488 nm).

Results and Discussion

Flow Cytometry Analysis of DNA. A DNA solution was dyed with a PicoGreen™ fluorescent probe. Figure 1a shows flow cytometric scattergrams of DNA at fluorescent intensities of 525 nm and 575 nm in a DNA solution containing PicoGreen™ with no added NaCl. Although the maximum emission wavelength of PicoGreen™ intercalated with DNA was 530 nm,[4] emission at 575 nm was also observed. This was due to a broad fluorescent spectrum of PicoGreen™ in the solution. Each dot in the images shown in Fig. 1a represents the emission pattern from aggregated DNA particles in the DNA solution. No significant particle size was observed in the buffer solution alone (less than 25 dots in 0.5 mL), whereas more than 450 dots were observed in 0.5 mL of a 1 ppb DNA solution. On the other hand, a large degree of emission at 525 nm and 575 nm was observed in a DNA solution. The emission from DNA at 525 nm was found to be much stronger than that at 575 nm. Therefore, a two-dimensional chart (i.e., intensity at 525 nm as x axis and intensity at 575 nm as y axis) contributes to determine a more accurate concentration of DNA in the solution than the intensity at the maximum emission wavelength of PicoGreen™ in the solution. Five gated regions (A–E) were defined, as shown in Fig. 1a, depending on the emission intensity at 525 nm and 575 nm. The particles in the gate-C region were defined as DNA molecules. Furthermore, no particles existed in the gate-C region when the buffer solution was analyzed by flow cytometry. The gate-D and E regions were established from flow cytometry analysis of a 5000 ppm γ-globulin solution containing no PicoGreen™. The dots in the gate-D and E regions were regarded as being negative control data, which originated from fluorescence of the aromatic rings of the proteins. The gate-B region was established from flow cytometry analysis of a 5000 ppm γ-globulin solution dyed with PicoGreen™. The dots in the gate-B regions were regarded as being aggregated γ-globulin. The gate-A region was remained regions from gate B–E. The color of the dots in the figure was determined from the regions where the dots were located (e.g., the dots in the gate-B region are shown as red dots in Figs. 1–3).

Figure 1b shows flow cytometric scattergrams of DNA at a fluorescent intensity of 525 nm, and the intensity of side light scattering of each DNA particle aggregate in the DNA solution containing PicoGreen™ with no NaCl added. A broad distribution of side light scattering was observed in the DNA solution, although the aggregated DNA particles showed a high intensity of emission at 525 nm, as shown in Fig. 1a. This result indicates that DNA aggregates in the DNA solution have different sizes and shapes, depending on each DNA aggregate.

Figure 2a shows flow cytometric scattergrams of a 5000 ppm γ-globulin solution containing PicoGreen™ with no added NaCl at fluorescent intensities of 525 nm and 575 nm. Each dot in the image shown in the figure represents the emission pattern for each particle of DNA or protein aggregate in the γ-globulin solution. The existence of particles was observed in not only the gate-C region, but also in gates B, D, and E. The particles in the gate-C region are ascribed to DNA contaminates in the γ-globulin solution, because DNA intercalated with PicoGreen™ showed the pattern in the gate-C region

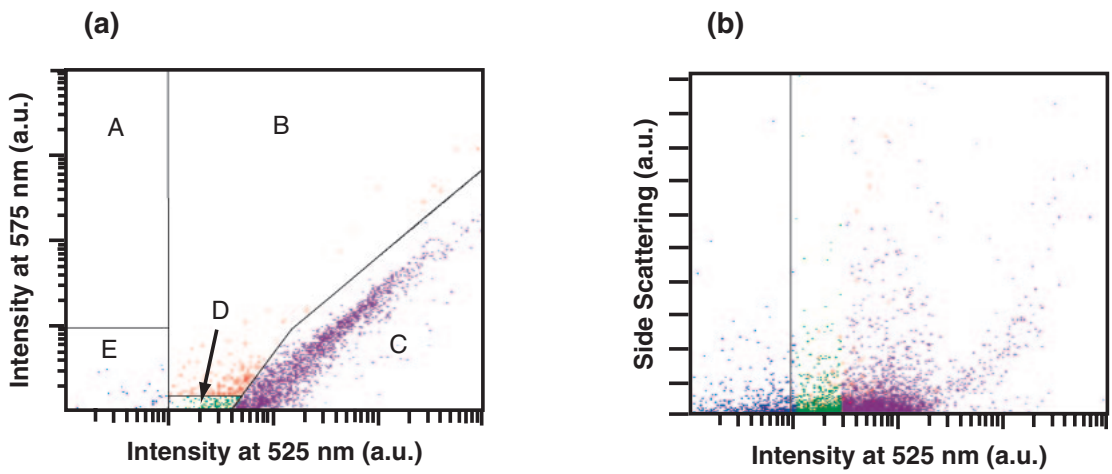

Fig. 1. Flow cytometric scattergrams of DNA solution containing PicoGreen™ at the fluorescent intensities of 525 nm and 575 nm (a) and at the fluorescent intensity of 525 nm and intensity of side light scattering (b) in 50 ppb DNA solution with no added NaCl. Typical data performed from four measurements ($n = 4$) under the same conditions were shown in this figure.

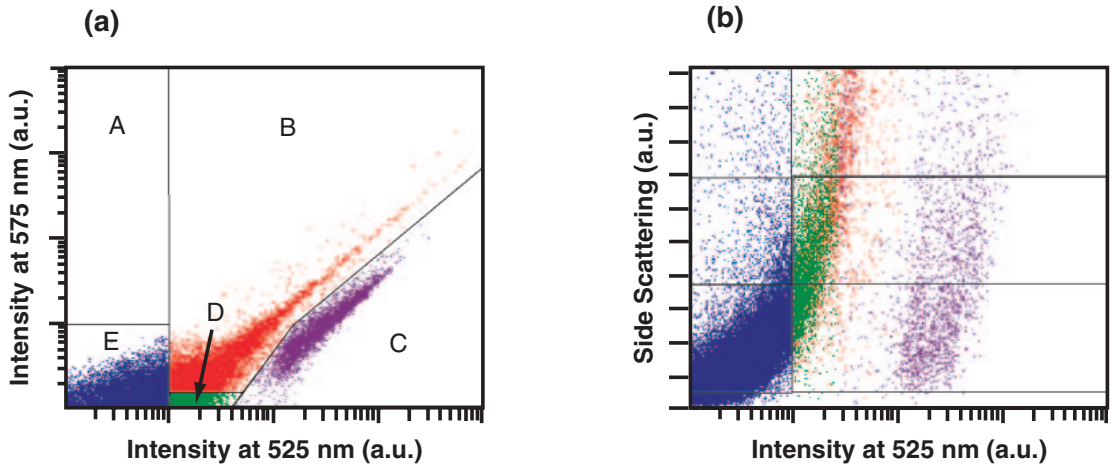

Fig. 2. Flow cytometric scattergrams of 5000 ppm γ-globulin solution containing PicoGreen™ with no added NaCl at the fluorescent intensities of 525 nm and 575 nm (a) and at the fluorescent intensity of 525 nm and intensity of side light scattering (b). Typical data performed from four measurements ($n = 4$) under the same conditions were shown in this figure.

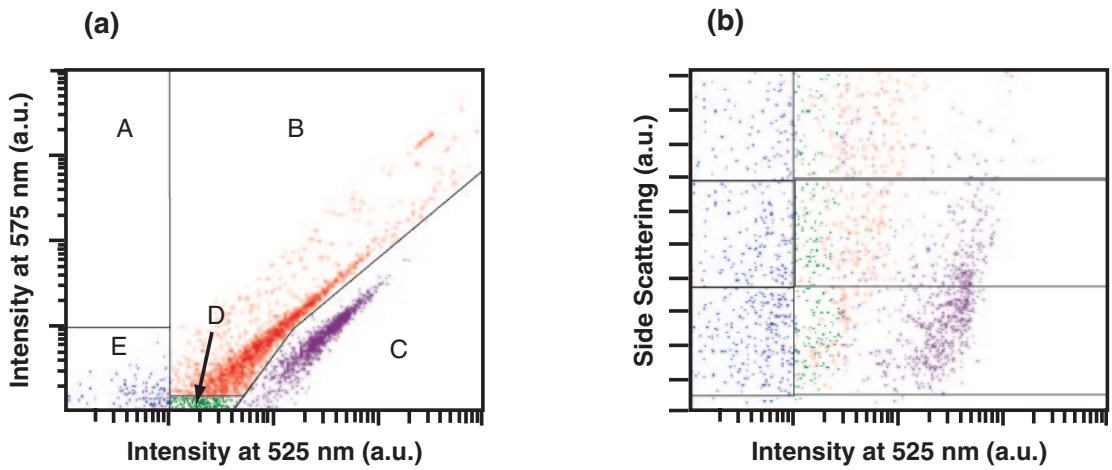

Fig. 3. Flow cytometric scattergrams of 5000 ppm γ-globulin solution containing PicoGreen™ and 0.15 mol/L of NaCl at the fluorescent intensities of 525 nm and 575 nm (a) and at the fluorescent intensity of 525 nm and intensity of side light scattering (b). Typical data performed from four measurements ($n = 4$) under the same conditions were shown in this figure.

as observed in Fig. 1a. The particles in the gate-B region are ascribed to aggregated proteins, both specifically and nonspecifically bound with PicoGreen™, because hydrophobic chemicals, such as fluorescent probes, tend to bind to the hydrophobic sites of proteins. Therefore, the quantitation of DNA in the protein solution is thought to be possible from a flow cytometry analysis of protein solutions dyed with a fluorescent probe.

Figure 2b shows flow cytometric scattergrams of a 5000 ppm γ-globulin solution containing PicoGreen™ with no added NaCl at a fluorescent intensity of 525 nm and intensity of side light scattering for each aggregated DNA particle and aggregated γ-globulins in the γ-globulin solution. There are two broad distributions of side light scattering at low and high fluorescent intensities at 525 nm, which are ascribed to γ-globulin aggregate and DNA aggregate contamination to the γ-globulin solution. These results indicate that DNA and γ-globulin aggregates in the γ-globulin solution have different sizes and shapes, depending on the individual aggregate. Further, the quantitation of DNA in the protein solution is thought to be possible from flow cytometric scattergrams of protein solutions at a fluorescent intensity of 525 nm and the intensity of side light scattering from flow cytometric analyses of protein solutions dyed with fluorescent probes.

Effect of NaCl on the Flow Cytometric Scattergrams. Figure 3a shows flow cytometric scattergrams of a 5000 ppm γ-globulin solution containing PicoGreen™ and 0.15 mol/L NaCl at fluorescent intensities of 525 nm and 575 nm. The flow cytometric scattergrams showed almost the same trends compared with that of a 5000 ppm γ-globulin solution with no added NaCl, although the numbers of aggregated γ-globulin and DNA were reduced in Fig. 3a, as compared with Fig. 2a.

Figure 3b shows flow cytometric scattergrams of a 5000 ppm γ-globulin solution containing PicoGreen™ and 0.15 mol/L NaCl at a fluorescent intensity of 525 nm and intensity of side light scattering for DNA particles and γ-globulin aggregates in the γ-globulin solution. There were two broad distributions for the side light scattering at low and high fluorescent intensities at 525 nm, which were ascribed to γ-globulin

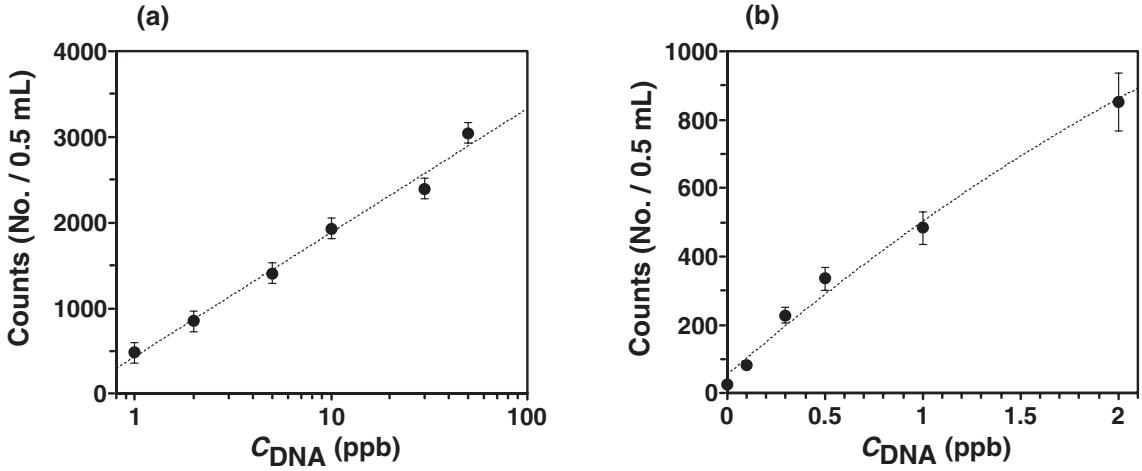

Fig. 4. Calibration curves of DNA concentration from flow cytometry analysis of DNA solution containing PicoGreen™ with no added NaCl. High concentration range of DNA standard curve (a) and low concentration range of DNA standard curve (b). Data are expressed as the means ± S.D. of four independent measurements.

and DNA aggregate contamination to the γ-globulin solution, although the numbers of aggregated γ-globulins and aggregated DNA were reduced in Fig. 3b relative to Fig. 2b.

The sizes and numbers of γ-globulin aggregates in the γ-globulin solution were reported to decrease from light-scattering measurements of γ-globulin and permeation measurements of γ-globulin through the membranes when the γ-globulin solution contained more than 0.05 mol/L NaCl.[4,5] These results were in good agreement with the decreased numbers of aggregated γ-globulin and DNA in the presence of NaCl observed in Figs. 3a and 3b.

DNA Quantitation from Flow Cytometry Analysis. DNA quantitation from flow cytometry analysis at fluorescent intensities of 525 nm and 575 nm that correspond to the gate-C region, as defined in Figs. 1a, 2a, and 3a, was performed using a 0.1 to 50 ppb calf thymus DNA solution containing PicoGreen™ in two standard curves (Fig. 4). The high standard curve showed a semi-logarithmic plot from 1 to 50 ppb of DNA with a correlation coefficient of 0.993 (Fig. 4a), which can be described by the following equation, when no NaCl was added to the DNA solution:

$$\text{Counts (No./0.5 mL)} = 1446.1 \times \log(C_{DNA}/\text{ppb}) + 438.23. \quad (1)$$

The lower standard curve was described by a second-power equation from 0.1 to 5 ppb DNA with a correlation coefficient of 0.998 (Fig. 4b); it is described by the following equation when no NaCl was added into the DNA solution:

$$\text{Counts (No./0.5 mL)} = -44.34 \times (C_{DNA}/\text{ppb})^2 + 492.4 \times (C_{DNA}/\text{ppb}) + 54.71. \quad (2)$$

These results were similar to those previously reported[1,4] using fluorescent spectroscopy for the measurements, although their calibration curves were described as linear equations.

The non-linear calibration curves (convex) for the standard curve for the DNA concentration shown in Fig. 4 is thought to be because DNA tends to coagulate at high DNA concentrations, and flow cytometry counts more aggregated DNA molecules as single particles in DNA solutions with higher DNA concentrations.

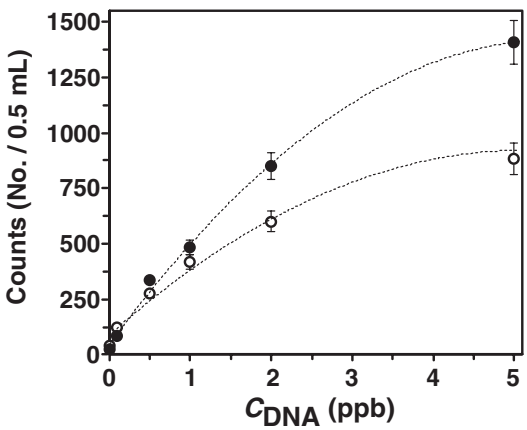

Fig. 5. Calibration curves of DNA concentration from flow cytometry analysis of DNA solution containing PicoGreen™ with no added NaCl (●) and containing PicoGreen™ and 0.15 mol/L NaCl (○). Data are expressed as the means ± S.D. of four independent measurements.

The calibration curves for the DNA concentration were also examined in a DNA solution containing PicoGreen™ with no NaCl and with 0.15 mol/L NaCl in an effort to investigate the effect of NaCl on the sensitivity of DNA detection from flow cytometry analysis. Figure 5 shows the calibration curves of the DNA concentration from a flow cytometry analysis of a DNA solution with no added NaCl, and that containing 0.15 mol/L NaCl. When NaCl was added to the DNA solution, the standard curve also showed a second-power equation from 0.1 to 5 ppb of DNA with a correlation coefficient of 0.995, as determined from the standard curve of Eq. 2, where NaCl was not added to the DNA solution. This curve could be described by the following equation:

$$\text{Counts (No./0.5 mL)} = -31.52 \times (C_{DNA}/\text{ppb})^2 + 324.1 \times (C_{DNA}/\text{ppb}) + 88.79. \quad (3)$$

Therefore, the counts of DNA particles in the flow cytometry analysis of the DNA concentration were reduced to 76% of the control value (those detected in the DNA solution containing no NaCl) for a 1 ppb DNA solution, and 63% of the control value for a 5 ppb DNA solution, compared to those in a DNA solution with no added NaCl, when 0.15 mol/L NaCl was added to the DNA solution.

The fluorescent-based DNA detection method using fluorescent spectroscopy was reported to demonstrate that the slope of the linear DNA standard curve measured in a DNA solution containing 0.15 mol/L NaCl declined to 12% of the control slope obtained from the DNA solution containing no NaCl.[1] This indicates that the sensitivity of DNA detection was reduced to 1/8.3 in the fluorescent-based DNA detection method, while the flow cytometry analysis presented in this study showed less than a 37% decline when a DNA solution of less than 5 ppb contained 0.15 mol/L NaCl. However, the calibration curves for the DNA concentration should also be established under each condition, depending on the concentration of salts in the solution analyzed from flow cytometry.

DNA Concentration in Protein Solution. DNA concentrations in 500–5000 ppm albumin, cellulase, γ-globulin, pepsin, and trypsin solutions were extrapolated from the calibration curves of Eqs. 1 and 2, and are summarized in Table 1. The DNA content in albumin, cellulase, γ-globulin, and pepsin was estimated to be 0.078 ± 0.006, 0.26 ± 0.04, 3.0 ± 0.4, and 1.2 ± 0.1 μg-DNA/g-protein ($n = 4$), respectively, from a flow cytometry analysis of the protein solutions. These results indicate that most of the proteins contain a trace amount of DNA, although the DNA content in trypsin was not detected in this study. γ-Globulin and pepsin were determined to contain relatively higher amounts of DNA than other proteins evaluated in this study. The DNA concentration in a 5000 ppm γ-globulin solution was estimated to be 8–25 ppb, depending on the preparation time for the γ-globulin solution based on conventional fluorescence measurements in previous studies.[4,5] The DNA concentration in the 500 ppm γ-globulin solution was determined to be 1.5 ppb, which corresponds to 15 ppb in the 5000 ppm γ-globulin solution. The present flow cytometry analysis used to determine the DNA concentration was determined to be in good agreement with that determined from the fluorescence measurements.

Table 1. Concentration of DNA in Protein Solution and Content of DNA in Protein

Proteins	Concentration of protein/ppm	Concentration of DNA/ppb	Content of DNA (μg-DNA/g-protein)
Albumin	5000	0.39 ± 0.03	0.078 ± 0.006
Cellulase	5000	1.3 ± 0.2	0.26 ± 0.04
γ-Globulin	500	1.5 ± 0.2	3.0 ± 0.4
Pepsin	5000	5.8 ± 0.5	1.2 ± 0.1
Trypsin	5000	<0.1	<0.02

Conclusion

DNA quantitation from flow cytometry analysis at fluorescent intensities of 525 nm and 575 nm, corresponding to the gate-C region, was successfully reproduced for 0.1–50 ppb DNA solutions prepared from calf thymus DNA in two standard curves. The flow cytometry analysis for the DNA concentration is expected to be a powerful means for estimating the DNA concentration of a protein solution containing salts, such as saline, because the present flow cytometry analysis showed less than a 37% decline when a less than 5 ppb DNA solution contained 0.15 mol/L NaCl. The sensitivity of DNA detection in the fluorescent-based DNA detection method reported in literature,[1] however, was reduced to 1/8.3. The DNA contents in albumin, cellulase, γ-globulin, and pepsin were estimated by flow cytometry analysis of the protein solutions to be 0.078 ± 0.006, 0.26 ± 0.04, 3.0 ± 0.4, and 1.2 ± 0.1 μg-DNA/g-protein ($n = 4$), respectively. These results indicate that most of the evaluated proteins contained trace amounts of DNA. The reproducible values for DNA concentrations were measured in the protein solutions using flow cytometry analysis in this study.

We gratefully acknowledge financial support through a Grant-in-Aid for Scientific Research on Priority Areas (B, "Novel Smart Membranes Containing Controlled Molecular Cavity", No. 13133202, 2001–2004) from the Ministry of Education, Culture, Sports, Science and Technology. This research was also supported by the Salt Science Foundation.

References

1 R. Bolger, F. Lenoch, E. Allen, B. Meiklejohn, and T. Burke, *BioTechniques*, **23**, 532 (1997).

2 A. Higuchi, K. Kato, M. Hara, T. Sato, G. Ishikawa, H. Nakano, S. Satoh, and S. Manabe, *J. Membr. Sci.*, **116**, 191 (1996).

3 A. Higuchi, T. Yoshimura, K. Kato, M. Hara, M. Higuchi, N. Minoura, T. Hirasaki, T. Sato, G. Ishikawa, H. Nakano, S. Satoh, and S. Manabe, *J. Membr. Sci.*, **126**, 7 (1997).

4 A. Higuchi, A. Komuro, K. Hirano, B. O. Yoon, M. Hara, T. Hirasaki, Y. Nishimoto, M. Yokogi, and S. Manabe, *J. Membr. Sci.*, **186**, 9 (2001).

5 A. Higuchi, M. Nemoto, H. Koyama, K. Hirano, B. O. Yoon, N. Hara, M. Yokogi, and S. Manabe, *J. Membr. Sci.*, **210**, 369 (2002).

6 V. T. Kung, P. R. Panfili, E. L. Sheldon, R. S. King, P. A. Nagainis, Jr., B. Gomez, D. A. Ross, J. Briggs, and R. F. Zuk, *Anal. Biochem.*, **187**, 220 (1990).

7 J. Briggs and P. R. Panfili, *Anal. Chem.*, **63**, 850 (1991).

8 E. Fabrizio, A. Nadim, and J. D. Sterling, *Anal. Chem.*, **75**, 5012 (2003).

9 A. Higuchi, S. Yamamiya, B. O. Yoon, M. Sakurai, and M. Hara, *J. Biomed. Mater. Res.*, **68A**, 34 (2004).

10 A. Higuchi and Y. Tsukamoto, *J. Biomed. Mater. Res.*, in press.