Product Application Focus

Development and Characterization of the NanoOrange[®] Protein Quantitation Assay: A Fluorescence-Based Assay of Proteins in Solution

Laurie J. Jones, Richard P. Haugland, and Victoria L. Singer Molecular Probes, Eugene, OR, USA

BioTechniques 34:850-861 (April 2003)

ABSTRACT

We developed a sensitive fluorescence assay for the quantitation of proteins in solution using the NanoOrange® reagent, a merocyanine dye that produces a large increase in fluorescence quantum yield upon interaction with detergent-coated proteins. The NanoOrange assay allowed for the detection of 10 ng/mL to 10 µg/mL protein with a standard fluorometer, offering a broad, dynamic quantitation range and improved sensitivity relative to absorption-based protein solution assays. The protein-to-protein variability of the NanoOrange assay was comparable to those of standard assays, including Lowry, bicinchoninic acid, and Bradford procedures. We also found that the NanoOrange assay is useful for detecting relatively small proteins or large peptides, such as aprotinin and insulin. The assay was somewhat sensitive to the presence of several common contaminants found in protein preparations such as salts and detergents; however, it was insensitive to the presence of reducing agents, nucleic acids, and free amino acids. The simple assay protocol is suitable for automation. Samples are briefly heated in the presence of dye in a detergent-containing diluent, allowed to cool to room temperature, and fluorescence is measured using 485-nm excitation and 590-nm emission wavelengths. Therefore, the NanoOrange assay is well suited for use with standard fluorescence microplate readers, fluorometers, and some laser scanners.

INTRODUCTION

Detecting and quantitating proteins is a fundamental step in numerous biochemical applications. Accordingly, there is great interest in developing sensitive, accurate, and reliable

850 BioTechniques

assays of proteins in solution, which are useful over a wide range of protein concentrations. Protein-to-protein variability, interference from contaminants, and the requirement for carefully timed reagent additions and incubations are common limitations of protein quantitation assays. Several absorption-based assays are widely employed for protein determinations; each method has advantages and limitations. The bicinchoninic acid (BCA) assay detects changes in 562 nm absorption caused by the formation of a purple-colored reaction product that results when Cu²⁺ is reduced and then chelated with BCA in the presence of proteins under alkaline conditions (1). The BCA assay is compatible with detergents and organic solvents but is not compatible with reducing agents. Also, the BCA assay requires absorbance measurements within 10 min for reliable results. The Bradford assay is based on the shift in absorption maximum of Coomassie Brilliant Blue® dye from 465 to 595 nm upon binding to protein (2). Because Coomassie Brilliant Blue dye binds specific amino acids and protein tertiary structures, the Bradford assay is somewhat protein-selective; however, the method is rapid and useful when the accuracy is not crucial. Reducing agents do not interfere with the Bradford assay, but detergents are not well tolerated. Using 750 nm absorption, the Lowry assay measures the production of a blue-colored reaction product that forms when Cu²⁺ is reduced to Cu⁺ at a high pH in the presence of proteins, the Cu⁺-chelating biuret reagent, and the color-enhancing Folin-Ciocalteu reagent (3). This method exhibits relatively low protein-toprotein variability but is not compatible with detergents, sugars, salts, or reducing agents. The procedure is also lengthy, with carefully timed steps. Ultraviolet light absorption at 280 nm is a rapid, nondestructive method for the quantitation of proteins in solution, based on the content of the aromatic amino acid residues, tyrosine and tryptophan. As a result, the assay sensitivity is highly protein selective, and nucleic acids and free amino acids interfere. In addition, because the extinction coefficients of these amino acids are fairly low, the assay is insensitive.

In principle, fluorescence-based techniques offer higher sensitivity, lower background signals, and wider dynamic ranges than absorbance-based techniques. There are two general methods for fluorescence-based protein detection. These methods employ either (*i*) nonfluorescent, reactive dyes that couple with protein amines to form fluorescent, covalent adducts or (*ii*) dyes that exhibit fluorescence enhancement upon non-covalent interaction with hydrophobic regions of proteins or detergent-coated proteins (4).

Amine-reactive fluorescamine (5), 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) (6), and ophthaldialdehyde (OPA) (7) reagents offer an excellent sensitivity and dynamic range for proteins and lipoproteins in solution. Fluorescamine is nonfluorescent but reacts with lysine and other primary amines to form fluorescent adducts that are detectable using 390-nm excitation and 475-nm emission wavelengths. CBQCA is nonfluorescent until it binds to primary aliphatic amines in the presence of cyanide or thiols. The resulting fluorescent products are measurable using 450-nm excitation and 550-nm emission wavelengths (6). OPA is also nonfluorescent, but, in the presence of 2mercaptoethanol (2-ME), it forms fluorescent adducts that are detectable using 340-nm excitation and 455-nm emission wavelengths (7). None of these reagents can be used with Tris, glycine, or other amine-containing buffers, and the presence of free amino acids or other materials containing primary amines interferes with detection. The sensitivities of these assays are dependent on the number of amines present on the protein in question; thus, they are very protein-selective. Therefore, although they can be excellent indicators for selected proteins in solution, their protein-toprotein variability limits their utility in assays of crude or mixed proteins.

Coating proteins with detergent as a means of improving fluorescence-based detection and quantitation has been used for the detection of proteins in solutions, gels, and capillary electrophoresis (8–14). Because certain ionic detergents coat proteins with near uniformity (9), protein quantitation methods that are based on detecting detergent-protein complexes should be generally more useful than methods based solely on the detection of primary amines. Nile red dye (9-ethylamino-5H-benzo[α]-phenoxazine-5-one) has been used for the detection of detergent-protein complexes in solution and in polyacrylamide gels (8,9). Over the past several years we also developed a series of dyes that are essentially nonfluorescent in aqueous solution but become intensely fluorescent upon binding to detergent-coated proteins or hydrophobic regions of proteins (10–15).

Our goal for these experiments was to develop a convenient, sensitive assay to measure small amounts of protein in solution, with a broad dynamic range, relative insensitivity to contaminants commonly found in protein preparations, a simple assay procedure that's amenable to automation (and avoids carefully timed steps), and minimal variation of fluorescence emission signals per unit mass for a wide variety of proteins. To achieve this goal, we screened a series of environmentally sensitive dyes (15) and selected the one that provided the greatest sensitivity, dynamic range, and reliability for detecting proteins in solution. Here we describe the development and characterization of the NanoOrange[®] protein quantitation assay that resulted from that screen.

MATERIALS AND METHODS

Materials

NanoOrange protein quantitation reagent, assay diluent, bungarotoxin, rabbit IgG, NeutrAvidin[™], streptavidin, Nile red, fluorescein, and rhodamine B were from Molecular Probes (Eugene, OR, USA). BSA (99% pure by SDS-PAGE) was from Calbiochem (San Diego, CA, USA). The Modified Lowry Protein Assay kit and the Micro BCA Protein Assay kit were from Pierce Chemical (Rockford, IL, USA). All other proteins and reagents were from Sigma (St. Louis, MO, USA).

Methods

Spectral characterization. Fluorescence excitation and emission spectra were determined for the NanoOrange reagent in assay diluent (10 mM Tris-HCl, pH 7.5, with a proprietary mixture of anionic detergents, including SDS) in the presence of 150 µg/mL BSA in 2 mL using a SLM AMINCO[®] SPF 500C[™] Spectrofluorometer (SLM Instruments, Urbana, IL, USA). Samples for fluorescence quantum yield (QY) determinations were prepared by incubating the NanoOrange reagent or Nile red with 300 µg/mL BSA, or buffer only, in 10 mM Tris-HCl, pH 7.5, 0.05% SDS for 30 min. OY measurements were made for NanoOrange reagent at 480 nm excitation, comparing the integrated fluorescence emissions of samples containing the NanoOrange reagent to the integrated fluorescence of fluorescein (QY, 0.92 in 50 mM phosphate buffer, pH 9.0) at equal dye absorbance at 480 nm. Rhodamine B (QY, 0.68 in methanol) was the standard for the samples containing Nile red, with an absorbance and excitation at 540 nm.

Protein quantitation. Working NanoOrange reagent solution was prepared by diluting the dye 500-fold into assay diluent, as described in the manufacturer's protocol. A 2-mg/mL stock solution of BSA in water was prepared and stored at 4°C. For each sample, the BSA solution was diluted into a solution of the NanoOrange reagent in assay diluent, using 2.5 mL for 13-mm disposable glass test tubes or 250 μ L for microfuge tubes, with a no protein sample included as a background fluorescence control. To prevent photobleaching, all samples were protected from light throughout the procedure. The samples were heated for 10 min at 96°C in a VWR heating block and cooled for 20 min at room temperature without light. The samples were mixed briefly and then transferred to disposable acrylic cuvettes (2 mL volumes) or acrylic microplate wells (200 μ L volumes).

BioTechniques 851

Fluorescence emission intensities were determined using 485-nm excitation and 590-nm emission settings or filters. Fluorescence was measured using an SLM AMINCO SPF500C Spectrofluorometer, a TD700 filter fluorometer (Turner Designs, Sunnyvale, CA, USA), or a CytoFluor 2350 fluorescence microplate reader (PerSeptive Biosystems, Foster City, CA, USA), with a 485 \pm 10 nm excitation filter and a 590 \pm 15 nm emission filter.

Comparison of the protein-to-protein variability of the NanoOrange assay with that of other protein quantitation assays. Stock solutions of proteins were prepared at 2 mg/mL in water or PBS and stored at 4°C. Protein samples were diluted into NanoOrange working solution in final volumes of 250 μ L in microfuge tubes, with a buffer-only sample included as a control. The samples were heated for 10 min at 96°C and cooled at room temperature for 20 min without light. After briefly vortex mixing, 200 μ L each sample were transferred to a 96-well microplate for measurement as described earlier. Modified Lowry and BCA protein assays were performed according to instructions supplied by the manufacturer. The Coomassie-based protein assay was performed as previously described (2).

Effects of additives on assay results. Samples containing 2–20 µg/mL BSA in 200 µL assay diluent were prepared. A diluent-only sample was prepared as a control. Stock solutions of additives were prepared at 10× final desired concentration in water, and 40 µL each 10× additive solution were added to each BSA sample. Control sample sets were made using 40 µL water. The NanoOrange reagent was diluted 200-fold in assay diluent, and 160 µL of this solution were added to each sample. All samples were then incubated in darkness at 96°C for 10 min and cooled in darkness at room temperature for 20 min. After briefly vortex mixing, 200 µL each sample were transferred to a 96-well microplate, and the fluorescence was measured.

sponse with that of Nile red. The QY of NanoOrange reagent was 0.36 when complexed with BSA in 0.05% SDS, which was much higher than that of Nile red under the same conditions (QY, 0.20). Furthermore, NanoOrange reagent's QY increased more than 200-fold upon forming a protein-detergent-dye complex, with negligible intrinsic fluorescence of the free dye in 0.05% SDS, while the relatively high background fluorescence of Nile red only contributed to an approximate 30-fold increase for that dye.

Simplicity of Assay Format

The NanoOrange assay protocol was easy to perform. Protein samples were added to diluted NanoOrange reagent, and the mixtures were heated briefly to approximately 95°C



Figure 1. Normalized fluorescence excitation (dashed curve) and emission spectra (solid curve) for the NanoOrange reagent complexed with $150 \ \mu g/mL BSA$ in protein quantitation diluent.



Figure 2. Dynamic range and sensitivity of the NanoOrange protein quantitation assay. (A) The indicated amounts of BSA were assayed with the NanoOrange reagent using the standard protocol (see Materials and Methods). The fluorescence emission intensity at 590 nm, following excitation at 485 nm, determined using a fluorometer was plotted versus BSA concentration. (B) Enlargement of the results from BSA concentrations ranging from 0 to $0.5 \,\mu g/mL$.

RESULTS AND DISCUSSION

Spectral Characterization of NanoOrange Reagent Bound to Detergent and Protein

We found that NanoOrange reagent-detergent-protein complexes have broad fluorescence excitation and emission spectra (Figure 1). In the presence of excess protein, the excitation maximum was between 470 and 490 nm (emission, 600 nm), and the emission maximum was between 570 and 590 nm (excitation, 485 nm). The emission maximum shifted to slightly longer wavelengths at lower protein concentrations. For example, for 10 ng/mL BSA, we found that the emission maximum was approximately 605 nm, while for 10 µg/mL BSA, the emission maximum was approximately 582 nm. We also compared the NanoOrange reagent's fluorescence re-



Figure 3. Protein-to-protein variability. Using the NanoOrange assay, dilutions of proteins were assayed using the standard protocol, and the signals obtained with a fluorescence microplate reader were plotted versus the protein concentration for each sample: BSA (stars), chicken egg albumin (open squares), rabbit IgG (open circles), horse myoglobin (open triangles), and NeutrAvidin (open diamonds), β -lactoglobulin (closed squares), β -lactoglobulin A (closed circles), carbonic anhydrase (closed triangles), and streptavidin (closed diamonds).



Figure 4. Comparison of protein-to-protein variability for the NanoOrange assay relative to other protein quantitation assays. (A) NanoOrange, (B) BCA, (C) Bradford, and (D) Lowry assays were compared using dilutions of the following proteins: BSA (stars), chicken egg albumin (open squares), rabbit IgG (open circles), horse myoglobin (open triangles), and NeutrAvidin (open diamonds). The assays were performed according to previously described procedures. Fluorescence measurements for the NanoOrange assay were obtained using a microplate reader. Absorption measurements were performed using a spectrophotometer for the BCA, Bradford, and Lowry assays (562, 595, and 750 nm, respectively).

854 BioTechniques

and cooled to room temperature. After cooling, fluorescence measurements could be made immediately or up to 6 h later with no loss of signal provided that the samples were protected from light. Therefore, NanoOrange reagent and its detergent-protein complex exhibited good chemical stability. The cooling step was essential. Warm samples yielded highly variable fluorescence signals and poor sensitivity. We found that the NanoOrange reagent is subject to photobleaching in dilute aqueous solution; therefore, we protected the samples from light as much as possible throughout each experiment. In addition, we found that it was important to use the same illumination durations for each assay to achieve optimal reproducibility. For a single experiment with triplicate samples, average coefficients of variation were typically 2%-5%, and the standard deviation error bars generally fell close to or within the plotted data points. The assay was therefore relatively robust. Although both heating and cooling steps are included, we found that as long as those steps are carried out for at least the minimum times that are recommended and the samples are handled in parallel, the timing is not critical. Thus, unlike most others, this protein assay is readily amenable to automation, and the reagents are reasonably stable.

Dynamic Range, Sensitivity, and Accuracy of the NanoOrange Assay

We found the NanoOrange assay to be extremely sensitive and reliably and accurately detected as little as 10 ng/mL BSA with a standard fluorometer (Figure 2). This sensitivity was 50to 100-fold greater than that obtained from Bradford, Lowry, or BCA assays, or absorption measurements at 280 nm. Using a fluorescence microplate reader, we found that as little as 100 ng/mL protein could be detected in a 200-µL volume, which yielded a sensitivity of 20 ng BSA per sample. The upper limit of the assay's dynamic range was approximately 10 µg/mL protein using either a microplate reader or fluorometer. Spanning three orders of magnitude, this dynamic range is comparable to or greater than those of absorbance-based assays. The response modeled well with a four-parameter sigmoidal fit over the full assay range, with typical data sets generating standard curves with correlation coefficients (r^2) greater than 0.996. For less than 1 µg/mL protein, linear and sigmoidal fits were equally appropriate. In addition, we found that different mixtures of anionic detergents extended the dynamic range to higher protein concentrations when one uses higher concentrations of NanoOrange reagent.

Vol. 34, No. 4 (2003)

Increasing the concentration of longer chain detergents in the mixture was especially helpful (data not shown). However, using the commercially available NanoOrange diluent with higher NanoOrange reagent concentrations gave rise to extremely nonlinear results.

Modest increases in Bradford assay sensitivity can be achieved by adding very small levels of Triton® X-100 (16-19), SDS (16,19) or phenol (20). Elevating the temperature improves avidin and strepavidin quantitation (21). However, the standard Bradford protein assay has been found to indicate lower protein concentrations than other assays (21-23). In contrast, the BCA assay (1) generally yields high estimates of protein concentration (3,24). A modification of the BCA assay, in which 2% SDS is added to the samples before analysis, has been found to reduce this overestimation for plasma lipoprotein concentrations (24). A different modification of the BCA assay, in which phosphotungstic acid is used to precipitate proteins, improves its accuracy (25). Several modifications of the original Lowry assay (3) have been developed to simplify the procedure (26-28). However, none of these modifications extended the sensitivity of any of these assays into the low nanogram region. Only the fluorescence-based CBQCA protein quantitation assay has comparable sensitivity to the NanoOrange assay, and it has much larger dynamic range, detecting 10 ng to 150 µg BSA (6). The utility of the CBQCA assay, however, is limited by the fact that it works by detecting primary amines. This assay and others that detect primary amines suffer from high protein-to-protein variability because there is substantial variability in primary amine content among proteins.

Protein-to-Protein Signal Variability

All protein quantitation assays show some protein-to-protein variability. Because ionic detergents can form complexes with proteins with a nearly constant mass ratio, detecting a detergent-protein complex should be more useful for general protein quantitation than detecting a specific protein functional group. To examine the protein-to-protein variability of the NanoOrange assay, we tested its response to a variety of proteins (Figure 3). To compare the NanoOrange assay response to those of other quantitation methods, we selected five of those proteins and applied the Lowry, Bradford, and BCA assays (Figure 4). We chose proteins with a wide range of properties. The two albumins are relatively hydrophobic. IgG is heavily glycosylated, while Neutr-Avidin has relatively low glycosylation. Myoglobin has a chromophoric prosthetic group. In addition, the proteins varied in molecular weight from approximately 18-150 kDa. For these five proteins, the modified Lowry assay exhibited the lowest protein-to-protein variability, and the Bradford assay showed the highest protein-to-protein variability. The BCA and Bradford assays showed the highest variation in background signals. When we performed a background subtraction for each protein dilution series, the protein-to-protein variability in the BCA assay appeared as good as, or better than, that of the Lowry assay. The NanoOrange assay showed low variation in background fluorescence and moderate protein-to-protein variability.

Table 1. Tolerance Levels for Contaminants in the NanoOrange Assay	
Compound	Maximum Tolerable Concentration
DTT	100 mM
2-ME	100 mM
Tween 20	<0.001%
Triton X-100	0.001%
SDS	0.01%
sodium chloride	20 mM
potassium chloride	20 mM
magnesium chloride	1 mM
calcium chloride	1 mM
zinc chloride	0.5 mM
sodium acetate	20 mM
sodium azide	10 mM
sodium phosphate	20 mM
ammonium sulfate	10 mM
HEPES	10 mM
EDTA	5 mM
sodium hydroxide	10 mM
hydrochloric acid	10 mM
ascorbic acid	10 mM
polyethylene glycol	1%
glycerol	10%
sucrose	10 mM
urea	1 M
DNA	100 ng/mL
amino acids	10 µg/mL



Figure 5. Detection of small proteins with the NanoOrange assay. The signals obtained from aprotinin (6.5 kDa; diamonds) and insulin (5.8 kDa; squares) were compared to those obtained from BSA (66 kDa; stars) using a fluorescence microplate reader.

Proteomic Technologies



858 BioTechniques

Figure 6. Effects of contaminants on the NanoOrange assay response. The indicated compounds were incubated at various concentrations with NanoOrange reagent in the presence of the indicated concentrations of BSA. Fluorescence was measured using a microplate reader. In all panels, closed stars represent no additive. (A) 0.1 (circles), 1 (triangles), 10 (squares), and 100 mM (diamonds) DTT; (B) 0.1 (circles), 1 (triangles), 10 (squares), and 100 mM (diamonds) 2-ME; (C) 0.001% (squares) and 0.01% (circles) Tween-20; (D) 0.001% (squares) and 0.01% (circles) Triton X-100; (E) 0.001% (squares) and 0.01% (circles) SDS; (F) 5 (open circles), 10 (open triangles), 20 (closed squares), 30 (open diamonds), 40 (closed circles), and 50 mM (open squares) NaCl; (G) 0.1 (open circles), 1 (open triangles), 5 (closed squares), 10 (open dia-monds), 20 (closed circles), and 50 mM (open squares) KCl; (H) 0.2 (squares) and 1 mM (circles) MgCl₂; (I) 1 (squares) and 5 mM CaCl₂ (circles); (J) 0.1 (squares) and 0.5 mM (circles) ZnCl₂; (K) 20 (squares) and 50 mM (circles) sodium acetate; (L) 10 (squares) and 100 mM (circles) sodium azide; (M) 20 (squares) and 50 mM (circles) sodium phosphate; (N) 10 (squares) and 100 mM (circles) ammonium sulfate; (O) 10 (squares) and 100 mM (circles) HEPES; (P) 5 (squares) and 50 mM (circles) EDTA; (Q) 10 (squares) and 100 mM (circles) NaOH; (R) 10 (squares) and 100 mM (circles) HCl; (S) 10 (squares) and 100 mM (circles) ascorbic acid; (T) 0.1% (squares) and 1% (circles) polyethylene glycol; (U) 1% (squares) and 10% (circles) glycerol; (V) 10 (squares) and 100 (circles) sucrose; (W) 10 (circles), 100 mM (triangles), and 1 M (squares) urea; (X) 10 (open squares) and 100 ng/mL (open circles) DNA overlaid with 1 µg/mL (closed squares) and 10 $\mu g/mL$

Vol. 34, No. 4 (2003)

In the NanoOrange assay, some acidic proteins such as pepsin showed lower fluorescence compared to BSA, while basic proteins such as lysozyme showed higher fluorescence (data not shown). This variability is probably related to differences in the binding of these proteins to the detergents contained in the NanoOrange diluent because positively charged proteins are likely to bind anionic detergents to a greater extent than are negatively charged proteins. Although heating proteins in diluent was not found to be essential for detecting relatively hydrophobic proteins such as BSA, we found that step to be important for reducing protein-to-protein signal variability and for achieving consistent results. However, small (approximately 10°C) fluctuations in temperature did not cause variations in the assay results. Proteins such as BSA that have hydrophobic binding sites or large hydrophobic regions sometimes exhibited higher fluorescence in this assay if the heating step was omitted because the native protein is able to bind dye more efficiently than the denatured protein. In particular, this would be the case for proteins that might have specific binding sites for dyes, such as the NanoOrange reagent. However, even for BSA, the assay consistency was improved by heating because the heating step denatured all the proteins in the sample, eliminating variability due to the presence of varying amounts of native protein. In addition, proteins such as IgG, which exhibit low fluorescence if the heating step is omitted, typically showed an increased fluorescence response after heating as a result of increased detergent and dye binding to the denatured protein. The choice of detergents in the diluent and their total and relative concentrations were also critical. Above the critical micelle concentration, the detergent itself binds dye, which causes high background fluorescence. However, concentrations of detergents that are too low do not thoroughly coat proteins (particularly proteins with low hydrophobicity), which limits the sensitivity of the assay and increases protein-to-protein variability.

The NanoOrange assay can also be used to detect relatively small proteins or large peptides. Figure 5 shows a comparison of standard curves generated using the NanoOrange assay for insulin, aprotinin, and BSA (5.8, 6.5, 66 kDa, respectively). These results illustrate the importance of using pure samples of the target protein when generating standard curves. However, the relatively low variability of the NanoOrange assay still makes it well suited for the quantitation of protein concentrations of protein mixtures, crude cell or tissue extracts, and for measuring concentrations of proteins of unknown amino acid composition.

Effects of Solution Components and Additives

We examined the performance of the NanoOrange assay in the presence of various concentrations of additives, including reducing agents, detergents, salts, sugars, acids, bases, and other compounds commonly used in protein preparations. Table 1 summarizes our estimates of the assay tolerance levels for the tested compounds. We defined the assay tolerance level for each additive as the highest tested concentration that resulted in less than or equal to a 20% change in fluorescence signal intensity. The relative contributions of these additives to the NanoOrange assay response using a BSA standard dilution series are shown in Figure 6. An important advantage of the NanoOrange assay is its tolerance for the presence of reducing agents. We found that the reducing agents DTT and 2-ME were well tolerated (Figure 6, panels A and B). Previously, the Bradford assay was considered the best option for use with reducing agents (14) despite its poor sensitivity and low accuracy. In contrast, detergents caused appreciable interference with the NanoOrange assay (Figure 6, panels C-E). Because the diluent already contains detergents, the addition of detergents can produce micelle formation and give rise to high background fluorescence. Tween® 20 and Triton X-100 caused considerable deviation at concentrations as low as 0.001%. Somewhat smaller but noticeable deviation was caused by 0.01% SDS. High concentrations of lipids interfere (data not shown), but they can be removed from protein solutions by acetone precipitation, followed by treatment with diethyl ether.

Salts were tolerated at low concentrations. High concentrations of some salts produced increases in background fluorescence and altered responses. Concentrations of sodium chloride and potassium chloride above 20 mM resulted in appreciable deviation. Both background fluorescence and the slopes of the standard curves were altered at elevated concentrations (Figure 6, panels F and G). The divalent salts, magnesium chloride, calcium chloride, and zinc chloride, were tolerated in the assay at lower concentrations (Figure 6, panels H–J). Sodium acetate, sodium azide, sodium phosphate, and ammonium sulfate increased assay background fluorescence, and above 10–20 mM, interference was dramatic (Figure 6, panels K–N).

HEPES buffer did not interfere at relatively low concentration (10 mM), but 100 mM HEPES caused a large increase in the background fluorescence and deviation from control measurements (Figure 6, panel O). Similarly, EDTA caused little interference at relatively moderate levels (5 mM), but background fluorescence limited the assay response at 50 mM EDTA (Figure 6, panel P). Figure 6, panels Q-S, shows the effects of acids and bases. Sodium hydroxide and hydrochloric acid caused considerable interference at concentrations above 10 mM. Ascorbic acid, which is also used as a reducing agent, caused no detectable interference at 10 mM. A variety of other additives, including glycerol, sucrose, or polyethylene glycol, did not interfere (Figure 6, panels T–V). Urea was also well tolerated, even at very high concentrations (Figure 6W). Nucleic acids and amino acids had little effect (Figure 6X). In general, one can compensate for the effects of additives by simply preparing a set of standards that contain the same concentration of contaminants that is present in the experimental samples. Furthermore, the high sensitivity of the assay makes it possible to dilute most potential contaminants to acceptable levels.

In summary, we developed a protein assay that overcomes many common limitations of standard assays. Its high sensitivity, broad dynamic range, low protein-to-protein variability, relative insensitivity to assay contaminants, simplicity of protocol, relatively stable reagents, and lack of carefully timed steps make it easy to use and compatible with existing instrumentation and automation. The NanoOrange reagent has recently been used to develop a human serum albumin assay for clinical diagnostics, in combination with a microplate reader and capillary electrophoresis (29). We think it is likely that the NanoOrange reagent will also prove useful for assaying other clinically important proteins in the future.

ACKNOWLEDGMENTS

The authors thank Tom Steinberg for his initial work on this project. We also thank Rakar West for graphic assistance and Peter O'Day for contributions to the manuscript.

REFERENCES

- Smith, P.K., R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, et al. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76-85.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.
- 3.Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.
- 4.Haugland, R.P. 1996. Detection of quantitation of proteins in solution, p. 180-185. *In* M.T.Z. Spence (Ed.), Handbook of Fluorescence Probes and Research Chemicals, 6th ed. Molecular Probes, Eugene, OR.
- 5.Udenfriend, S., S. Stein, P. Bohlen, W. Dairman, W. Leimgruber, and M. Weigele. 1972. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. Science 178:871-872.
- 6.You, W.W., R.P. Haugland, D.K. Ryan, and R.P. Haugland. 1997. 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde, a reagent with broad dynamic range for the assay of proteins and lipoproteins in solution. Anal. Biochem. 244:277-282.
- 7.Benson, J.R. and P.E. Hare. 1975. O-phthalaldehyde: fluorogenic detection of primary amines in the picomole range. Comparison with fluorescamine and ninhydrin. Proc. Natl. Acad. Sci. USA 72:619-622.
- Baban, J.R., S. Bartolome, and M. Samso. 1991. Use of the hydrophobic probe nile red for the fluorescent staining of protein bands in sodium dodecyl sulfate-polyacrylamide gels. Anal. Biochem. 199:169-174.
- Daban, J.R., M. Samso, and S. Bartolome. 1991. Use of nile red as a fluorescent probe for the study of the hydrophobic properties of proteinsodium dodecyl sulfate complexes in solution. Anal. Biochem. 199:162-168.
- Patton, W.F. 2000. Making blind robots see: the synergy between fluorescent dyes and imaging devices in automated proteomics. BioTechniques 28:944-957.
- 11.Singer, V.L. and R.P. Haugland. 1999. Fluorescent imaging of nucleic acids and proteins in gels, p. 51-62. *In* W.T. Mason (Ed.), Fluorescent and Luminescent Probes for Biological Activity, 2nd ed. Academic Press, London.
- Steinberg, T.H., R.P. Haugland, and V.L. Singer. 1996. Applications of SYPRO orange and SYPRO red protein gel stains. Anal. Biochem. 239:238-245.
- 13.Steinberg, T.H., L.J. Jones, R.P. Haugland and V.L. Singer. 1996. SYPRO orange and SYPRO red protein gel stains: one-step fluorescent staining of denaturing gels for detection of nanogram levels of protein. Anal. Biochem. 239:223-237.
- 14.Steinberg, T.H., W.M. Lauber, K. Berggren, C. Kemper, S. Yue, and W.F. Patton. 2000. Fluorescence detection of proteins in sodium dodecyl sulfate-polyacrylamide gels using environmentally benign, nonfixative, saline solution. Electrophoresis 21:497-508.
- 15. Haugland, R.P., V.L. Singer, L.J. Jones, and T.H. Steinberg, inventors; Molecular Probes, assignee. Non-specific protein staining using merocyanine dyes. US patent 5 616 502. 1997 Apr 1.
- 16.Duhamel, R.C., E. Meezan, and K.J. Brendel. 1981. The addition of SDS to the Bradford dye-binding protein assay, a modification with in-

- creased sensitivity to collagen. J. Biochem. Biophys. Methods 5:67-74.
- Friedenauer, S. and H.H. Berlet. 1989. Sensitivity and variability of the Bradford protein assay in the presence of detergents. Anal. Biochem. 178:263-268.
- Loffler, B.M. and H. Kunze. 1989. Refinement of the Coomassie brilliant blue G assay for quantitative protein determination. Anal. Biochem. 177:100-102.
- Lopez, J.M., S. Imperial, R. Valderrama, and S. Navarro. 1993. An improved Bradford protein assay for collagen proteins. Clin. Chim. Acta 220:91-100.
- 20.Marshall, T. and K.M. Williams. 1986. Phenol addition to the Bradford dye binding assay improves sensitivity and gives a characteristic response with different proteins. J. Biochem. Biophys. Methods 13:145-150.
- Sharma, H.K. and C. Tihon. 1988. Determination of avidin and streptavidin by a modified Bradford method. Anal. Biochem. 170:135-139.
- 22.Kirazov, L.P., L.G. Venkov, and E.P. Kirazov. 1993. Comparison of the Lowry and the Bradford protein assays as applied for protein estimation of membrane-containing fractions. Anal. Biochem. 208:44-48.
- 23.Sharma, S.K. and J.A. Babitch. 1980. Application of Bradford's protein assay to chick brain subcellular fractions. J. Biochem. Biophys. Methods 2:247-250.
- 24.Morton, R.E. and T.A. Evans. 1992. Modification of the bicinchoninic acid protein assay to eliminate lipid interference in determining lipoprotein protein content. Anal. Biochem. 204:332-334.
- 25.Singh, B.P., S. Sridhara, N. Arora, and S.V. Gangal. 1992. Evaluation of protein assay methods for pollen and fungal spore extracts. Biochem. Intl. 27:477-484.
- 26.Bensadoun, A. and D. Weinstein. 1976. Assay of proteins in the presence of interfering materials. Anal. Biochem. 70:241-250.
- Ohnishi, S.T. and J.K. Barr. 1978. A simplified method of quantitating protein using the biuret and phenol reagents. Anal. Biochem. 86:193-200
- Peterson, G.L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83:346-356.
- 29.Harvey, M.D., V. Bablekis, P.R. Banks, and C.D. Skinner. 2001. Utilization of the non-covalent fluorescent dye, NanoOrange, as a potential clinical diagnostic tool. Nanomolar human serum albumin quantitation. J. Chromatogr. B 784:345-356.

Address correspondence to Laurie J. Jones, Molecular Probes, 29851 Willow Creek Road, Eugene, OR, USA. e-mail: laurie.jones@probes.com