

Proteomics products greatly streamline protocols for whole proteome analysis and promise to bring to proteomics the same capability for rapid, large-scale data acquisition that fluorescence has brought to genomics and other fields.

Molecular Probes' Reagents and Kits for Proteomics

In this chapter, Section 9.2 includes our reagents and kits (including the NanoOrange and CBQCA protein quantitation reagents) for quantitating proteins and certain protein modifications in solution. Section 9.3 includes our important SYPRO stains for detecting and quantitating total proteins in gels and on blots. Our unique reagents for Multiplexed Proteomics in Section 9.4 include several important products that permit qualitative and quantitative detection of specific proteins or modifications of proteins. Among these are the following products:

- DyeChrome Western Blot Stain Kits for simultaneous dichromatic staining of total proteins and specific proteins on blots (and probably microarrays)
- DyeChrome Double Western Blot Stain Kit (D-21887) — the first technology that permits simultaneous trichromatic detection of total proteins and two different specific proteins or protein modifications on a single Western blot

- Pro-Q Western Blot Stain Kits for staining of proteins on blots using alkaline phosphatase–conjugated reagents and DDAO phosphate
- Pro-Q Emerald 300 and Pro-Q Emerald 488 glycoprotein gel and blot stains — the world's best and easiest-to-use stains for detecting periodate-oxidized glycoproteins
- Amplex Gold Kits for Western Blots, for staining of horseradish peroxidase–labeled targets on Western blots
- Pro-Q Sapphire 365 and Pro-Q Sapphire 488 oligohistidine gel stains, for fast and easy detection of oligohistidine fusion proteins in gels
- The BOLD APB chemiluminescent substrate (B-21901) for ultrasensitive detection of alkaline phosphatase conjugates on PVDF or nitrocellulose membranes

Section 9.5 describes reagents used in the synthesis of fluorescent dye– or hapten-labeled peptides and fluorogenic protease substrates, as well as in peptide analysis and sequencing.

References

1. Proteomics 1, 169 (2001).

9.2 Quantitation and Selective Purification of Proteins in Solution

Several colorimetric methods have been described for quantitating proteins in solution, including the widely used Bradford¹ and Lowry² assays, as well as an assay described by Smith³ that uses bicinchoninic acid (BCA). However, because they rely on absorption-based measurements, these methods are inherently limited in sensitivity and effective range. Molecular Probes has developed two unique fluorometric methods for quantitating proteins in solution — the NanoOrange Protein Quantitation Kit (N-6666) and the CBQCA Protein Quantitation Kit (C-6667) — that outperform *all* existing methods (Table 9.1). We also offer several other fluorescent reagents useful for protein detection in solution.

NanoOrange Protein Quantitation Kit

Our patented NanoOrange Protein Quantitation Kit (N-6666) provides an ultrasensitive assay for measuring the concentration of proteins in solution.⁴ The NanoOrange Protein Quantitation Kit has several important features:

- **Ease of use.** The NanoOrange assay protocol is much easier to perform than the Lowry method (Figure 9.3). Protein samples are simply added to the diluted NanoOrange reagent, and the mixtures are heated at 95°C for 10 minutes. After cooling the mixtures to room temperature, their fluorescence emissions are measured directly. The interaction of proteins with the NanoOrange reagent produces a large fluorescence enhance-

ment that may be used to generate a standard curve for protein determination; fluorescence of the reagent in the absence of proteins is negligible.

- **Sensitivity and effective range.** The NanoOrange assay can detect proteins at a final concentration as low as 10 ng/mL when a standard spectrofluorometer or minifluorometer is used. A single protocol is suitable for quantitating protein concentrations between 10 ng/mL and 10 µg/mL — an effective range of three orders of magnitude (Figure 9.4).
- **Stability.** The NanoOrange reagent and its protein complex have high chemical stability. In contrast to the Bradford and BCA assays, readings can be taken for up to six hours after sample preparation with no loss in signal, provided that samples are protected from light.
- **Little protein-to-protein variability** (Figure 9.5). The NanoOrange assay is not only more sensitive, but shows less protein-to-protein variability than Bradford assays.
- **Insensitivity to sample contaminants.** Unlike the Lowry and BCA assays, the NanoOrange assay is compatible with the presence of reducing agents. Furthermore, the high sensitivity of the assay and stability of the protein–dye complex make it possible to dilute out most potential contaminants, including detergents and salts (Table 9.2). Nucleic acids do not interfere with protein quantitation using the NanoOrange reagent. Although unusually high concentrations of lipids in the sample can interfere with the NanoOrange assay, this interference can

be eliminated by acetone precipitation of the protein, followed by delipidation with diethyl ether.⁵

Our NanoOrange protein quantitation reagent is suitable for use with a variety of instrumentation. Fluorescence is measured using instrument settings or filters that provide excitation/emission at ~485/590 nm, which are commonly available for both spectrofluorometers and microplate readers. A spectrofluorometer — either a standard fluorometer or a minifluorometer — offers the greatest effective range and lowest detection limits for this assay. With microplate-based fluorescence readers, the NanoOrange assay is useful over a somewhat narrower range — from 100 ng/mL to 10 µg/mL in final protein concentration.

The NanoOrange Protein Quantitation Kit (N-6666) supplies:

- Concentrated NanoOrange reagent in dimethylsulfoxide (DMSO)
- Concentrated NanoOrange diluent
- Bovine serum albumin (BSA) as a protein reference standard
- A detailed protocol for protein quantitation

The amount of dye supplied in this kit is sufficient for ~200 assays using a 2 mL assay volume and a standard or minifluorometer, or ~2000 assays using a 200 µL assay volume and a fluorescence microplate reader.

The NanoOrange reagent is ideal for quantitating protein samples before gel electrophoresis⁵ and Western blot analysis.⁶ It has also been used to measure bound versus free protein levels in protein binding assays, and was even able to detect protein trapped in filters during a separation step.⁷ The NanoOrange reagent is also

Table 9.1 A comparison of reagents for detecting and quantitating proteins in solution.

| Assay | Detection Wavelengths (nm) * | Sensitivity and Effective Range | Mechanism of Action | Notes |
|--|------------------------------|---|--|--|
| NanoOrange protein quantitation assay (N-6666) | 485/590 | 10 ng/mL to 10 µg/mL | Binds to detergent coating on proteins and hydrophobic regions of proteins; the unbound dye is nonfluorescent | <ul style="list-style-type: none"> • High sensitivity • Little protein-to-protein variation • Rapid and accurate assay with a simple procedure • Compatible with reducing agents |
| CBQCA protein quantitation assay (C-6667) | 450/550 | 10 ng/mL to 150 µg/mL | Reacts with primary amine groups on proteins in the presence of cyanide or thiols; the unbound dye is nonfluorescent | <ul style="list-style-type: none"> • Sensitivity depends on the number of amines present • Not compatible with buffers containing amines or thiols • High sensitivity • Linear over an extended range of protein concentration |
| Bradford assay ¹ (Coomassie brilliant blue) | 595 | 1 µg/mL to 1.5 mg/mL | Directly binds specific amino acids and protein tertiary structures; the dye's color changes from brown to blue | <ul style="list-style-type: none"> • High protein-to-protein variation • Not compatible with detergents • Rapid assay • Useful when accuracy is not crucial |
| BCA method ² (bicinchoninic acid) | 562 | 0.5 µg/mL to 1.2 mg/mL | Cu ²⁺ is reduced to Cu ⁺ in the presence of proteins at high pH; the BCA reagent chelates Cu ⁺ ions, forming purple-colored complexes | <ul style="list-style-type: none"> • Compatible with detergents, chaotropes and organic solvents • Not compatible with reducing agents • The sample must be read within 10 minutes |
| Lowry assay ³ (biuret reagent plus Folin–Ciocalteu reagent) | 750 | 1 µg/mL to 1.5 mg/mL | Cu ²⁺ is reduced to Cu ⁺ in the presence of proteins at high pH; the biuret reagent chelates the Cu ⁺ ion, then the Folin–Ciocalteu reagent enhances the blue color | <ul style="list-style-type: none"> • Lengthy procedure with carefully timed steps • Not compatible with detergents or reducing agents |
| Fluorescamine ^{4–7} (F-2332, F-20261) | 390/475 | 0.3 µg/mL to 13 µg/mL | Reacts with primary amine groups on proteins; unbound dye is nonfluorescent | <ul style="list-style-type: none"> • Sensitivity depends on the number of amines present • Reagent is unstable • Not compatible with Tris or glycine buffers |
| OPA ^{8–10} (o-phthalaldehyde) (P-2331) | 340/455 | 0.2 µg/mL to 25 µg/mL | Reacts with primary amine groups on proteins in the presence of β-mercaptoethanol; unbound dye is nonfluorescent | <ul style="list-style-type: none"> • Sensitivity depends on the number of amines present • Not compatible with Tris or glycine buffers • Low cost |
| UV absorption ¹¹ | 205/280 | 10 µg/mL to 50 µg/mL or 50 µg/mL to 2 mg/mL | Peptide bond absorption; tryptophan and tyrosine absorption | <ul style="list-style-type: none"> • Sensitivity depends on number of aromatic amino acid residues present • Nondestructive • Low cost |

* Excitation and emission wavelength maxima or absorption wavelength maximum, in nm.

1. Anal Biochem 72, 248 (1976); 2. Anal Biochem 150, 76 (1985); 3. J Biol Chem 193, 265 (1951); 4. Science 178, 871 (1972); 5. Clin Chim Acta 157, 73 (1986); 6. J Lipid Res 27, 792 (1986); 7. Anal Biochem 214, 346 (1993); 8. Anal Biochem 115, 203 (1981); 9. Biotechniques 4, 130 (1986); 10. J Immunol Methods 172, 141 (1994); 11. Protein Purification: Principles and Practice, 2nd Ed., Scopes RK, pp. 253–283 (1987).

an optimal reagent for detecting proteins that have been separated by microchip capillary electrophoresis.^{8,9} A high-throughput assay has been developed for quantitating human serum albumin by both microplate and capillary electrophoresis laser-induced fluorescence (CE-LIF) that may be suitable for clinical samples.¹⁰ Additionally, the NanoOrange reagent has been shown to be useful in cell-based assays, including an assay designed to measure total protein content of cell cultures¹¹ and a rapid method for demonstrating flagellar movement of bacteria.¹²

CBQCA Protein Quantitation Kit

The ATTO-TAG CBQCA reagent was originally developed as a chromatographic derivatization reagent for amines^{13,14} (Section 1.8), but this reagent is also useful for quantitating proteins (Figure 9.6) by virtue of its rapid and quantitative reaction with their accessible amines. Molecular Probes has developed the CBQCA Protein Quantitation Kit (C-6667, Figure 9.7), which employs the ATTO-TAG CBQCA reagent for rapid and sensitive protein quantitation in solution¹⁵ (Table 9.1). The CBQCA protein quantitation assay functions well in the presence of lipids and detergents,^{15,16} substances that interfere with many other protein determination methods.¹⁵ For example, the CBQCA-based assay can be used directly to determine the protein content of lipoprotein samples

Table 9.2 Tolerance levels for contaminants in the NanoOrange protein quantitation assay.

| Contaminating Compound | Maximum Tolerable Concentration * |
|---|-----------------------------------|
| Glycerol | 10% by volume |
| Poly(ethylene glycol) (PEG) | 1% by volume |
| Urea | 1 M |
| Dithiothreitol (DTT), -mercaptoethanol | 100 mM |
| KCl, NaCl, sodium acetate, sodium phosphate | 20 mM |
| Ammonium sulfate, ascorbic acid, HEPES buffer, HCl, NaOH, sodium azide, sucrose | 10 mM |
| EDTA | 5 mM |
| Calcium chloride, magnesium chloride | 1 mM |
| Amino acids | 100 µg/mL |
| DNA | 100 ng/mL |
| Sodium dodecyl sulfate (SDS) | 0.01% |
| Tween 20, Triton X-100 | 0.001% |

* Compounds present in the final assay solution at or below the indicated concentrations do not appreciably interfere with the NanoOrange protein quantitation assay. Whenever feasible, the blank and protein standards should be prepared in a solution closely matching that of the experimental samples.

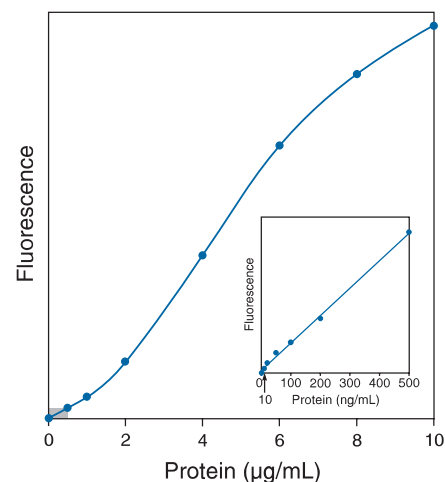
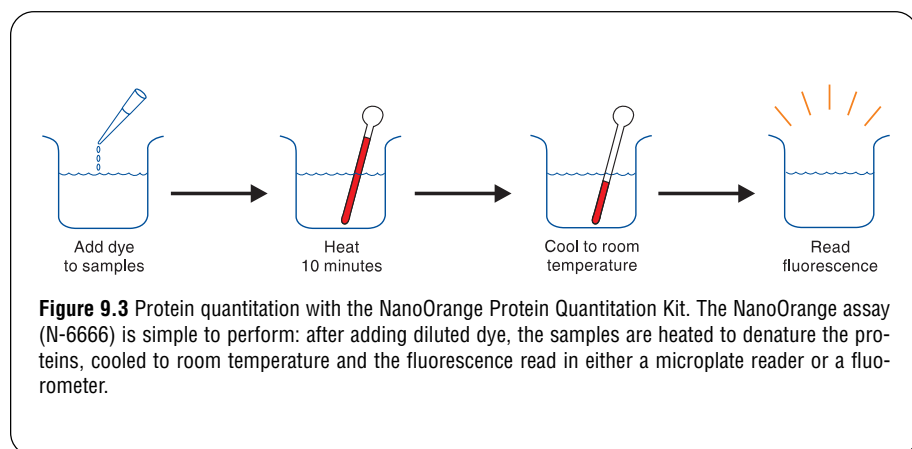


Figure 9.4 Quantitative analysis of bovine serum albumin (BSA) using the NanoOrange Protein Quantitation Kit (N-6666). Fluorescence measurements were carried out on an SLM SPF-500C fluorometer using excitation/emission wavelengths of 485/590 nm. The inset shows an enlargement of the results obtained (0–500 ng protein per mL) and illustrates the detection limit of ~10 ng/mL.

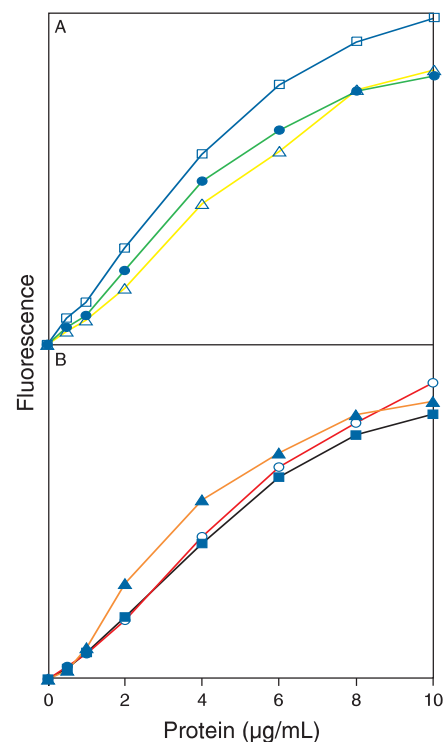


Figure 9.5 Quantitative analysis of six different proteins using the NanoOrange Protein Quantitation Kit (N-6666): Panel A) bovine serum albumin (BSA, □), trypsin (●) and carbonic anhydrase (△); Panel B) IgG (■), streptavidin (○) and RNase A (▲). The y-axis fluorescence intensity scale is the same in both panels, illustrating the minimal protein-to-protein staining variation of the NanoOrange assay. Data were collected on a microplate reader with excitation/emission wavelengths set at 485 ± 20/590 ± 35 nm.

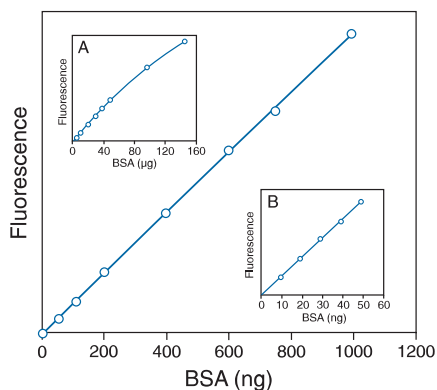


Figure 9.6 Detection of bovine serum albumin (BSA) using the CBQCA Protein Quantitation Kit (C-6667). The primary plot shows detection of BSA from 50 ng to 1000 ng. Inset A shows that the detection range can extend up to 150 µg. Inset B shows that the lower detection limit can extend down to 10 ng. Each point is the average of four determinations.

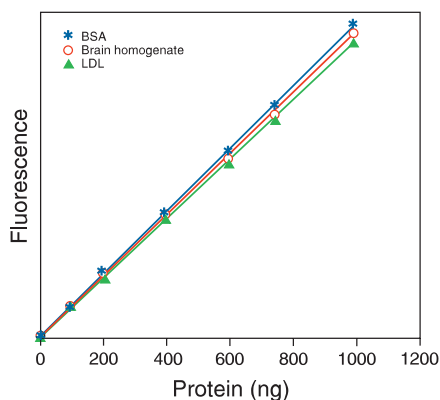


Figure 9.8 Quantitation of proteins in a lipid environment using the CBQCA Protein Quantitation Kit. The protein concentrations of an LDL preparation and a bovine brain homogenate were first determined by the modified Lowry method using BSA as a standard. Assays were then performed using the CBQCA Protein Quantitation Kit (C-6667) on samples containing from 100 ng to 1000 ng protein in 0.1 M sodium borate buffer, pH 9.3, containing 0.1% Triton X-100. Similar results were obtained without the addition of detergent (data not shown). Fluorescence was measured using a fluorescence-based microplate reader with excitation at 485 ± 10 nm and emission detection at 530 ± 12.5 nm. Each point is the average of three determinations.

The CBQCA protein quantitation assay is very easy to perform and has outstanding sensitivity, linearity and dynamic range.

or lipid–protein mixtures (Figure 9.8). The CBQCA assay has been shown to give faster and more sensitive detection of both free amino acids in human plasma¹⁷ and both low and high molecular weight primary amines in clinical samples from hemodialysis.¹⁸ ATTO-TAG CBQCA is more water soluble than either fluorescamine or *o*-phthalaldehyde and much more stable in aqueous solution than fluorescamine. Moreover, ATTO-TAG CBQCA provides greater sensitivity for protein quantitation in solution than either fluorescamine or *o*-phthalaldehyde (Figure 9.9). As little as 10 ng of BSA can be detected in a 100–200 µL assay volume using a fluorescence microplate reader, and the effective range extends up to 150 µg (Figure 9.6). Alternatively, the reaction mixtures can be diluted to 1–2 mL for fluorescence measurement in a standard fluorometer or mini-fluorometer.

Each CBQCA Protein Quantitation Kit (C-6667) contains:

- The ATTO-TAG CBQCA detection reagent
- Potassium cyanide
- Dimethylsulfoxide (DMSO)
- Bovine serum albumin (BSA) protein reference standard
- A detailed protocol for protein quantitation

The CBQCA Protein Quantitation Kit provides sufficient reagents for 300–800 assays using a standard fluorometer, minifluorometer or fluorescence microplate reader.

Other Reagents for Protein Quantitation in Solution

Most other fluorescent reagents for general protein quantitation in solution detect accessible primary amines. The sensitivity of assays based on these reagents therefore depends on the number of amines available — a function of both the protein's three-dimensional structure and its amino acid composition. For example, horseradish peroxidase (MW ~40,000 daltons), which has only six lysine residues,¹⁹ will be detected less efficiently than egg white avidin (MW ~66,000 daltons), which has 36 lysine residues,^{20,21} and bovine serum albumin (MW ~66,000 daltons), which has 59 lysine residues.²² However, the assays are generally rapid and easy to conduct, particularly in mini-fluorometer and fluorescence microplate reader formats.

Certain dyes that detect primary aliphatic amines, including ATTO-TAG CBQCA (A-6222), fluorescamine (F-2332; FluoroPure Grade, F-20261) and *o*-phthalaldehyde (OPA, P-2331), have been the predominant reagents for fluorometric determination of proteins in solution (Table 9.1). These same reagents, and others such as naphthalene-2,3-dicarboxaldehyde^{23,24} (NDA, N-1138, Section 1.8), have frequently been used for amino acid analysis of hydrolyzed proteins.

Fluorescamine

Fluorescamine (F-2332; FluoroPure Grade, F-20261) is intrinsically nonfluorescent but reacts in milliseconds with primary aliphatic amines, including peptides and proteins,

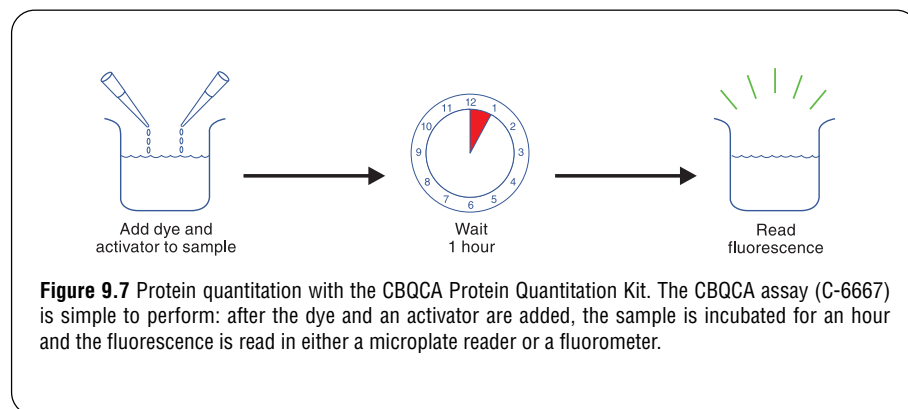


Figure 9.7 Protein quantitation with the CBQCA Protein Quantitation Kit. The CBQCA assay (C-6667) is simple to perform: after the dye and an activator are added, the sample is incubated for an hour and the fluorescence is read in either a microplate reader or a fluorometer.

to yield a fluorescent derivative²⁵ (Figure 1.102). This amine-reactive reagent has been shown to be useful for determining protein concentrations of aqueous solutions^{26–28} and for measuring the number of accessible lysine residues in proteins.²² Protein quantitation with fluorescamine is particularly well suited to a minifluorometer or fluorescence-based microplate reader.²⁹ Fluorescamine can also be used to detect proteins in gels and to analyze low molecular weight amines by TLC, HPLC and capillary electrophoresis.³⁰

o-Phthaldialdehyde

The combination of *o*-phthaldialdehyde (OPA, P-2331) and 2-mercaptoethanol provides a rapid and simple method of determining protein concentrations in the range of 0.2 µg/mL to 25 µg/mL³¹ (Figure 1.103). As compared to fluorescamine, OPA is both more soluble and stable in aqueous buffers and its sensitivity for detection of peptides is reported to be 5–10 times better.³² The OPA assay for lysine content is reasonably reliable over a broad range of proteins.²² OPA (and likely the ATTO-TAG CBQCA reagent) can also be used to detect *increases* in the concentration of free amines that result from protease-catalyzed protein hydrolysis.³³

SYPRO Red and SYPRO Orange Protein Gel Stains

An assay has been reported that uses the SYPRO Red protein gel stain (S-6653, S-6654; Section 9.3) for quantitating total protein content of bacterial cells by flow cytometry.³⁴ This assay provides an accurate measure of planktonic bacterial biomass in marine samples. Fluorescence of the SYPRO Orange protein gel stain (S-6650, S-6651; Section 9.3) has been used to follow isothermal protein denaturation.³⁵

Selective Protein Quantitation in Solution

MBDS: A Fluorogenic Reagent for Serum Albumins

4-Amino-4'-benzamido-stilbene-2,2'-disulfonic acid (MBDS, A-11760) is a reagent with properties similar to a commonly used probe for hydrophobic sites in proteins, 1-anilino-naphthalene-8-sulfonic acid (1,8-ANS, A-47; Section 13.5). Like 1,8-ANS (Figure 9.10; see Monitoring Protein-Folding Processes with Anilino-naphthalene-sulfonate Dyes in Section 13.5), MBDS is virtually nonfluorescent in water (quantum yield <0.01); however, upon binding to the hydrophobic pocket of serum albumins and some other proteins, it undergoes an almost 100-fold increase in its fluorescence.^{36,37}

Anti-Dinitrophenyl Antibody: A Reagent for Measuring Protein Carbonyls

Oxidative injury can be monitored by following the formation of protein-derived aldehydes and ketones. Traditionally, protein-derived aldehydes and ketones have been quantitated using a colorimetric assay based on their reaction with dinitrophenylhydrazine to yield protein-bound dinitrophenyl moieties (DNP). A much more sensitive ELISA method has been developed that detects the protein-bound DNP using unlabeled or biotin-labeled anti-DNP antibodies^{38,39} (A-6430, A-6435; Section 7.4). The bound anti-DNP antibody is subsequently detected with horseradish peroxidase-conjugated secondary detection reagents (Section 7.3). Our Alexa Fluor 488 and fluorescein conjugates of the anti-DNP antibody (A-11097, A-6423; Section 7.3) may potentially also be applied to this detection scheme. Our polyclonal antibody to nitrotyrosine (A-21285, Section 7.4) can be used similarly to separate and detect proteins of cell extracts that have been naturally nitrated by nitric oxide (Section 19.3, Figure 19.20). Use of these rabbit polyclonal antibodies in combination with Captivate ferrofluid goat anti-rabbit IgG antibody (C-21474, Section 7.3) permits selective isolation of modified proteins and their targets from solutions (Figure 7.62, Figure 7.94).

EnzChek and Amplex Red Assay Kits

Molecular Probes prepares numerous chromogenic and fluorogenic substrates that are useful for quantitating enzymes and enzymatic activity in experimental samples. In addition, we have developed several EnzChek Assay Kits and Amplex Red Assay Kits especially designed for detecting a wide variety of enzymes and their substrates. Most of these products are described in Chapter 10.

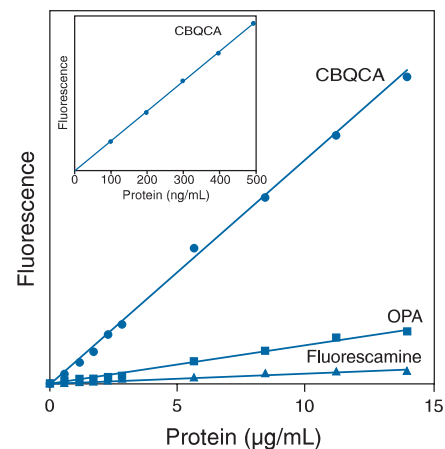


Figure 9.9 Comparison of the fluorometric quantitation of bovine serum albumin (BSA) using ATTO-TAG CBQCA (which is supplied in the CBQCA Protein Quantitation Kit, C-6667), OPA (P-2331) or fluorescamine (F-2332, F-20261). BSA samples were derivatized using large molar excesses of the fluorogenic reagents and were analyzed using a fluorescence-based microplate reader. Excitation/emission wavelengths were 360/460 nm for OPA and fluorescamine and 485/530 nm for ATTO-TAG CBQCA. The inset shows an enlargement of the results obtained using CBQCA to assay protein concentrations between 0 and 500 ng/mL.

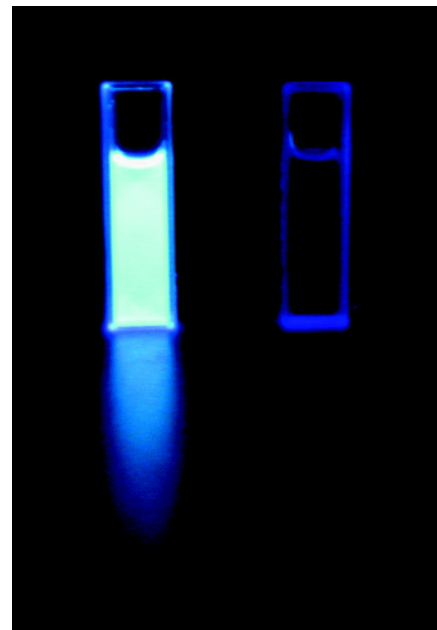


Figure 9.10 Fluorescence enhancement of 1,8-ANS (1-anilino-naphthalene-8-sulfonic acid, A-47) upon binding to protein. The image shows aqueous solutions of 1,8-ANS excited by ultraviolet light. The addition of protein (bovine serum albumin) to the solution in the cuvette on the left results in intense blue fluorescence. In comparison, the fluorescence of uncomplexed free dye in the cuvette on the right is negligible.

Selective Protein Purification

Glutathione Agarose and Anti-Glutathione S-Transferase Antibody for GST Fusion Protein Purification and Identification

In protein fusion techniques, the coding sequence of one protein is fused in-frame with another so that the expressed hybrid protein possesses desirable properties of both parent proteins. One common partner in these engineered products is glutathione S-transferase (GST), a protein with natural binding specificity that can be exploited to facilitate its purification.⁴⁰ Because the GST portion of the fusion protein retains its affinity and selectivity for glutathione, the fusion protein can be conveniently purified from the cell lysate in a single step by affinity chromatography on glutathione agarose^{41–46} (Figure 9.11). For purification of GST fusion proteins, Molecular Probes offers glutathione linked via the sulfur atom to crosslinked beaded agarose (10 mL of sedimented bead suspension, G-2879). This reagent is also available from Molecular Probes in bulk quantities (100 mL of sedimented bead suspension, G-21800). Each milliliter of gel can bind approximately 5–6 mg of bovine-liver GST. Adding excess free glutathione liberates the GST fragment from the matrix, which can then be regenerated by washing with a high-salt buffer.

Molecular Probes also offers a highly purified rabbit polyclonal anti-GST antibody (A-5800) that can be used to purify GST fusion proteins by immunoprecipitation.⁴⁷ This highly specific antibody, which was generated against a 260-amino acid N-terminal fragment of the *Schistosoma japonica* enzyme expressed in *Escherichia coli*, is also useful for detecting GST fusion proteins on Western blots (Section 9.4) and for detecting GST distribution in cells (Section 7.5). The intensely green-fluorescent Alexa Fluor 488 conjugate of anti-glutathione S-transferase (A-11131) is also available for direct detection of GST fusion proteins.

Our Glutathione Transferase Fusion Protein Purification Kit (G-21801) facilitates isolation and characterization of GST fusion proteins. This kit, which contains sufficient materials for five isolations, contains:

- Glutathione agarose
- Purification columns
- Anti-glutathione S-transferase antibody
- A detailed protocol

Following purification, the fusion protein can serve as an immunogen for antibody production^{48,49} or its properties can be compared with those of the native polypeptide to provide insights on the normal function of the polypeptide of interest. Such methods have been used to investigate biological properties of many proteins. Examples include cleavage of the capsid assembly protein ICP35 by the herpes simplex virus type 1 protease,⁵⁰ the role of the Rho GTP-binding protein in *lbc* oncogene function⁵¹ and the association of v-Src with cortactin in Rous sarcoma virus-transformed cells.⁵² In fact, the Ca²⁺-binding properties of a protein kinase C-GST fusion protein were examined while the GST fusion protein was still bound to the glutathione agarose.⁵³ Likewise, interactions of a DNA-binding protein-GST fusion protein have been assessed using an affinity column consisting of the fusion protein bound to glutathione agarose.⁴¹ Alternatively, the GST fusion expression vector can be engineered to encode a recognition sequence for a site-specific protease, such as thrombin or factor Xa, between the GST structural gene and gene of interest.^{54–57} Once the fusion protein is bound to the affinity matrix, the site-specific enzyme can be added to release the protein.

Streptavidin Agarose and CaptAvidin Agarose

Molecular Probes prepares both streptavidin and CaptAvidin biotin-binding protein conjugated to 4% beaded crosslinked agarose (S-951, C-21386) — matrices that can be used to isolate biotinylated peptides, proteins, hybridization probes, haptens and other molecules.⁵⁸ In addition, biotinylated antibodies can be bound to streptavidin agarose or CaptAvidin agarose to generate affinity matrices for the large-scale isolation of antigens.⁵⁸ For instance, streptavidin agarose has been used to isolate acetylcholine receptors from cultured myotubes after labeling the receptors with biotinylated α -bungarotoxin⁵⁹ (B-1196, Section 16.2). Streptavidin agarose has also been used to investigate the turnover of cell-surface proteins that had previously been derivatized with an amine-reactive biotin⁶⁰ (B-1582, Section 4.2). The binding capacity of our streptavidin agarose is mea-

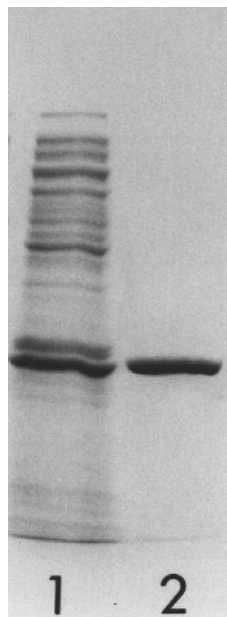


Figure 9.11 Coomassie brilliant blue-stained SDS-polyacrylamide gel, demonstrating the purification of a glutathione S-transferase (GST) fusion protein using glutathione agarose (G-2879, G-21800). Lane 1 contains crude supernatant from an *Escherichia coli* lysate and lane 2 contains the affinity-purified GST fusion protein.

TECHNICAL NOTE

Affinity Capture

Molecular Probes prepares several products that are useful for capturing specific proteins from complex mixtures. Most of these reagents are used in combination with a biotin- or DSB-X biotin-labeled antibody to the protein or a labeled lectin:

- Streptavidin agarose for essentially irreversible affinity capture of biotinylated probes or easily reversible capture of DSB-X biotin-labeled proteins
- Captivate ferrofluid superparamagnetic particles labeled with anti-mouse IgG or anti-rabbit IgG antibodies or streptavidin for reversible magnetic separation of target proteins (and cells)
- DSB-X biotin protein labeling and conjugate separation kits and DSB-X biotin conjugates for facile reversibility of biotin-avidin binding

sured in an assay using fluorescein biotin (B-1370, Section 4.3, Figure 4.10). Typically, the conjugate binds 15–20 μg (18–24 nanomoles) of fluorescein biotin per milliliter of sedimented gel. Our DSB-X biotin technology (see below) makes the capture and release of antigens and receptors from solutions even easier.

CaptAvidin agarose has been specially designed to allow easier dissociation of the avidin–biotin complex (Figure 7.85). Avidin and biotin form a very strong noncovalent bond with a K_a of $\sim 10^{15} \text{ M}^{-1}$. Although this high affinity is advantageous for many histochemical applications, it is a major drawback for affinity chromatography. The conditions needed to dissociate the avidin–biotin complex (8 M guanidine hydrochloride, pH 1.5) are usually too harsh for proteins and prevent the use of avidin for purifying biotinylated molecules. To address this problem, the tyrosine residues in the four biotin-binding sites of CaptAvidin biotin-binding protein (C-21385) are nitrated, considerably reducing its affinity for biotinylated molecules above pH 9. At pH 4.0, CaptAvidin biotin-binding protein binds biotin tightly, with a K_a of 10^9 M^{-1} . At pH 10, however, this association is reversed, allowing complete dissociation of the avidin–biotin complex.

Researchers have used CaptAvidin agarose affinity chromatography to purify immunoglobulins from whole rabbit serum and to isolate anti-transferrin antibody directly from rabbit IgG fractions.⁶¹ CaptAvidin agarose can be used to isolate cellular proteins that are selectively biotinylated with the reagents in our Fluoreporter Cell-Surface Biotinylation Kit (F-20650, Section 4.2) and to selectively isolate glycoproteins bound to the biotin-XX conjugate of concanavalin A (C-21420, Section 7.7). The biotin-binding capacity of CaptAvidin derivatives is at least 10 μg of biotin per mg protein.

Streptavidin Acrylamide, CaptAvidin Acrylamide and Reactive Acrylamide Derivatives

Streptavidin acrylamide (S-21379), which is prepared from the succinimidyl ester of 6-((acryloyl)amino)hexanoic acid (acryloyl-X, SE, A-20770; Section 5.2), may be useful for the preparation of biosensors.⁶² A similar streptavidin acrylamide has been shown to copolymerize with acrylamide on a polymeric surface to create a uniform monolayer of the immobilized protein. The streptavidin can then bind biotinylated ligands, including biotinylated hybridization probes, enzymes, antibodies and drugs. CaptAvidin acrylamide (C-21387) is expected to have similar utility, but offers an advantage — the bond that it forms with biotinylated probes should be reversible at about pH 10.

Like streptavidin and CaptAvidin biotin-binding protein, other amine-containing biomolecules can be crosslinked to acrylamides using acryloyl-X SE. Acryloyl-X SE reacts with amines of proteins, amine-modified nucleic acids and other biomolecules to yield acrylamides that can be copolymerized into polyacrylamide matrices or on surfaces, such as in microarrays and in biosensors.

DSB-X Biotin: Easily Reversible Binding to Streptavidin Agarose

Our exclusive DSB-X biotin technology, which is described in greater detail in Section 7.6, permits the selective binding and release of proteins that are labeled with DSB-X biotin succinimidyl ester, a component of our DSB-X Biotin Protein Labeling Kit (D-20655, Section 1.2). Temporary immobilization of a DSB-X biotin–conjugated macromolecule, such as an antibody, on streptavidin agarose permits the antibody to selectively capture antigens from solutions (Figure 7.89). Following gentle elution at

neutral pH with either D-biotin or D-desthiobiotin (Figure 4.1), the DSB-X biotin–conjugated protein and its targets are completely released, permitting further analysis of the released proteins (or nucleic acids) in gels or on blots.

The DSB-X Bioconjugate Isolation Kit #1 (D-20658) provides:

- Streptavidin agarose (5 mL of a sedimented bead suspension)
- Solutions of D-biotin or D-desthiobiotin
- Purification columns
- A suggested protocol for binding and release of DSB-X biotin conjugates

DSB-X biotin–labeled proteins can be prepared with the DSB-X Biotin Protein Labeling Kit (D-20655, Section 1.2).

Captivate Ferrofluid Conjugates

The Captivate ferrofluid conjugates of streptavidin (C-21476), goat anti–mouse IgG antibody (C-21473) and goat anti–rabbit IgG antibody (C-21474), which have been developed in cooperation with Immunicon Corporation, permit the facile isolation of biotinylated, DSB-X biotin–labeled or antibody-complexed proteins — including antibodies and their haptens, as well as receptors and their receptor ligands — using magnetic separation technology (Section 7.3, Section 7.6). Cells that have been selectively separated by the Captivate ferrofluid conjugates (Figure 7.94) can be lysed and analyzed for their proteins by standard gel electrophoresis (Section 9.3) or blotting techniques (Section 9.4). The Captivate ferrofluid streptavidin conjugate can also bind biotinylated lectins and DSB-X biotin–labeled lectins for selective isolation of glycoproteins from solutions.

The Captivate ferrofluid products are unique in that they represent the only superparamagnetic particles available that allow both cell sorting and cell-based imaging to be performed simultaneously by use of the Captivate microscope-mounted magnetic yoke assembly and associated Captivate disposable sample chambers (C-24700, C-24701; Section 24.3; Figure 7.62). The Captivate microscope-mounted magnetic yoke assembly includes one free set of 10 disposable sample chambers. Use of Captivate ferrofluid streptavidin in combination with biotin- or DSB-X biotin–conjugated probes permits the simultaneous isolation, visualization and counting of cells that are targets of the antibody by any researcher with access to a standard low-cost microscope with a 10 \times objective. Also, when used to capture DSB-X biotin–labeled antibodies to cell-surface antigens the Captivate ferrofluid can be completely separated from the labeled cells by incubation with D-biotin (B-1595, B-20656; Section 4.2) or D-desthiobiotin (D-20657, Section 4.2). The extremely fast capture rate and small particle size of Captivate ferrofluid means that these products should also have significant advantages over other commercially available magnetic particles in liquid-handling robotic systems.

References

1. Anal Biochem 72, 248 (1976);
2. J Biol Chem 193, 265 (1951);
3. Anal Biochem 150, 76 (1985);
4. FASEB J 10, A1512, abstract #2954 (1996);
5. J Biol Chem 272, 12762 (1997);
6. J Biol Chem 275, 3256 (2000);
7. J Biol Chem 274, 35367 (1999);
8. Anal Chem 73, 4994 (2001);
9. Anal Chem 72, 4608 (2000);
10. J Chromatogr B Biomed Sci Appl 754, 345 (2001);
11. J Cell Biol 142, 1313 (1998);
12. Appl Environ Microbiol 66, 3632 (2000);
13. Anal Chem 63, 413 (1991);
14. Anal Chem 63, 408 (1991);
15. Anal Biochem 244,

References — continued

- 277 (1997); **16.** J Biol Chem 274, 25461 (1999); **17.** J Chromatogr B Biomed Sci Appl 754, 217 (2001); **18.** Clin Chim Acta 308, 147 (2001); **19.** Eur J Biochem 96, 483 (1979); **20.** Adv Protein Chem 29, 85 (1975); **21.** J Biol Chem 246, 698 (1971); **22.** Anal Biochem 115, 203 (1981); **23.** Anal Chem 62, 1580 (1990); **24.** Anal Chem 62, 1577 (1990); **25.** Science 178, 871 (1972); **26.** Clin Chim Acta 157, 73 (1986); **27.** J Lipid Res 27, 792 (1986); **28.** Anal Biochem 248, 195 (1997); **29.** Anal Biochem 214, 346 (1993); **30.** J Chromatogr 502, 247 (1990); **31.** J Immunol Methods 172, 141 (1994); **32.** Proc Natl Acad Sci U S A 72, 619 (1975); **33.** Anal Biochem 123, 41 (1982); **34.** Appl Environ Microbiol 65, 3251 (1999); **35.** Anal Biochem 292, 40 (2001); **36.** J Biochem (Tokyo) 101, 89 (1987); **37.** Biochim Biophys Acta 229, 547 (1971); **38.** Free Radic Biol Med 23, 361 (1997); **39.** Methods Enzymol 300, 106 (1999); **40.** Proc Natl Acad Sci U S A 83, 8703 (1986); **41.** Meth Mol Genet 1, 267 (1993); **42.** Biotechniques 13, 856 (1992); **43.** Biotechniques 10, 178 (1991); **44.** Nucleic Acids Res 19, 4005 (1991); **45.** Science 252, 712 (1991); **46.** Gene 67, 31 (1988); **47.** J Biol Chem 272, 8133 (1997); **48.** J Cell Biol 131, 1003 (1995); **49.** J Cell Biol 130, 651 (1995); **50.** J Biol Chem 270, 30168 (1995); **51.** J Biol Chem 270, 9031 (1995); **52.** J Biol Chem 270, 26613 (1995); **53.** J Biol Chem 268, 3715 (1993); **54.** J Biol Chem 270, 24525 (1995); **55.** J Cell Biol 129, 189 (1995); **56.** Mol Biol Cell 6, 247 (1995); **57.** Biochemistry 31, 5841 (1992); **58.** J Chromatogr 510, 3 (1990); **59.** J Cell Biol 125, 661 (1994); **60.** Biochemistry 28, 574 (1989); **61.** Anal Biochem 243, 257 (1996); **62.** Anal Biochem 282, 200 (2000).

Data Table — 9.2 Quantitation and Selective Purification of Proteins in Solution

| Cat # | MW | Storage | Soluble | Abs | EC | Em | Solvent | Notes |
|---------|--------|---------|------------------|------|--------|------|---------|---------|
| A-6222 | 305.29 | F,D,L | MeOH | 465 | ND | 560 | MeOH | 1, 2, 3 |
| A-11760 | 518.47 | L | H ₂ O | 342 | 37,000 | 450 | pH 7 | |
| A-20770 | 282.30 | F,D,L | DMSO | <300 | | none | | |
| B-20656 | 244.31 | RO | pH >6 | <300 | | none | | 4 |
| D-20657 | 214.26 | RO | pH >6 | <300 | | none | | 4 |
| F-2332 | 278.26 | F,D,L | MeCN | 380 | 7,800 | 464 | MeCN | 5 |
| F-20261 | 278.26 | F,D,L | MeCN | 380 | 8,400 | 464 | MeCN | 5, 6 |
| P-2331 | 134.13 | L | EtOH | 334 | 5,700 | 455 | pH 9 | 7 |

For definitions of the contents of this data table, see "How to Use This Book" on page viii.

Notes

- Spectral data are for the reaction product with glycine in the presence of cyanide. Unreacted reagent in MeOH: Abs = 254 nm (EC = 46,000 cm⁻¹M⁻¹), nonfluorescent.
- ND = not determined.
- Solubility in methanol is improved by the addition of base (e.g., 1–5% (v/v) 0.2 M KOH).
- This product is supplied as a ready-made solution in the solvent indicated under **Soluble**.
- Fluorescamine spectra are for reaction product with butylamine. Fluorescence quantum yield/lifetime of adduct in EtOH are 0.23 and 7.5 nanoseconds, respectively (Arch Biochem Biophys 163, 390 (1974)). Unreacted reagent in MeCN: Abs = 234 nm (EC = 28,000 cm⁻¹M⁻¹), nonfluorescent.
- This product is specified to equal or exceed 98% analytical purity by HPLC.
- Spectral data are for the reaction product of P-2331 with alanine and 2-mercaptoethanol. The spectra and the stability of the adduct depend on the amine and thiol reactants (Biochim Biophys Acta 576, 440 (1979)). Unreacted reagent in H₂O: Abs = 257 nm (EC = 1000 cm⁻¹M⁻¹).

Product List — 9.2 Quantitation and Selective Purification of Proteins in Solution

| Cat # | Product Name | Unit Size |
|---------|---|-----------|
| A-20770 | 6-((acryloyl)amino)hexanoic acid, succinimidyl ester (acryloyl-X, SE) | 5 mg |
| A-11760 | 4-amino-4'-benzamido stilbene-2,2'-disulfonic acid, disodium salt (MBDS) | 100 mg |
| A-5800 | anti-glutathione S-transferase, rabbit IgG fraction *3 mg/mL* | 0.5 mL |
| A-11131 | anti-glutathione S-transferase, rabbit IgG fraction, Alexa Fluor® 488 conjugate *2 mg/mL* | 0.5 mL |
| A-6222 | ATTO-TAG™ CBQCA derivatization reagent (CBQCA; 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde) | 10 mg |
| B-20656 | o-biotin *50 mM aqueous solution* | 10 mL |
| C-21387 | CaptAvidin™ acrylamide | 1 mg |
| C-21386 | CaptAvidin™ agarose *sedimented bead suspension* | 5 mL |
| C-21385 | CaptAvidin™ biotin-binding protein | 1 mg |
| C-21473 | Captivate™ ferrofluid goat anti-mouse IgG (H+L) *0.5 mg Fe/mL* | 1 mL |
| C-21474 | Captivate™ ferrofluid goat anti-rabbit IgG (H+L) *0.5 mg Fe/mL* | 1 mL |
| C-21476 | Captivate™ ferrofluid streptavidin (streptavidin, Captivate™ ferrofluid conjugate) *0.5 mg Fe/mL* | 1 mL |
| C-6667 | CBQCA Protein Quantitation Kit *300–800 assays* | 1 kit |
| D-20657 | o-desthiobiotin *50 mM aqueous solution* | 10 mL |
| D-20658 | DSB-X™ Bioconjugate Isolation Kit #1 *with streptavidin agarose* *5 isolations* | 1 kit |
| F-2332 | fluorescamine | 100 mg |
| F-20261 | fluorescamine *FluoroPure™ grade* | 100 mg |
| G-2879 | glutathione agarose, linked through sulfur *sedimented bead suspension* | 10 mL |
| G-21800 | glutathione agarose, linked through sulfur *sedimented bead suspension* *bulk packaging* | 100 mL |
| G-21801 | Glutathione Transferase Fusion Protein Purification Kit *5 purifications* | 1 kit |
| N-6666 | NanoOrange® Protein Quantitation Kit *200–2000 assays* | 1 kit |
| P-2331 | o-phthalaldehyde (OPA) *high purity* | 1 g |
| S-21379 | streptavidin acrylamide | 1 mg |
| S-951 | streptavidin agarose *sedimented bead suspension* | 5 mL |