Molecular Probes™ Handbook
A Guide to Fluorescent Probes and Labeling Technologies

CHAPTER 9
Protein Detection and Analysis

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# 9.1 Introduction to Protein Detection

## Proteomics: A Rapidly Developing Field

For decades, polyacrylamide gel electrophoresis and related blotting techniques have formed the core technologies for protein analysis. Traditionally, these technologies have been paired with chromogenic dye–based protein detection techniques, such as silver or Coomassie brilliant blue staining. With the rapid growth of proteomics, however, the limitations and experimental disadvantages of absorption-based detection technologies and labor-intensive silver staining techniques have become glaringly apparent. The field of proteomics requires new, highly quantitative electrophoresis and blotting techniques that can interface seamlessly with improved microanalysis methods and that can perform in an increasingly high-throughput environment. These requirements are particularly important for quantitative proteomics and multiplexing techniques.

Fluorescence- or luminescence-based detection technologies offer the opportunity for multicolor labeling, making multiplexed analysis possible (Figure 9.1.1). In particular, Molecular Probes’ fluorescent and luminescent protein stains enable the identification of specific protein modifications (for example, phosphorylation, glycosylation and epitope tags) within the context of the entire protein profile. Simultaneous measurement of several variables greatly increases the amount of data that can be collected in a single experiment. In addition, directly comparing multiple measurements leads to more controlled experiments, more accurate data and fewer ambiguities. The detection characteristics of our protein stains greatly streamline protocols for 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® Detection Technology for Proteomics

We are meeting the demands of the rapidly expanding field of proteomics through the development of fluorescence- and luminescence-based detection methods for proteins in solutions and on gels, blots and microarrays. We are continuing to develop new reagents and detection methods for proteins and their modifications, such as phosphorylation, glycosylation and epitope tags, as well as improved methods of separating and analyzing peptides and proteins. Our advanced technologies are compatible with modern needs for sensitivity, specificity, sequencing compatibility and automatability. Application of our unique detection reagents requires minimal investment in labor, as compared with older technologies, while significantly increasing throughput, reducing total cost and improving accuracy. Furthermore, the greater sensitivity and linearity of most of our premier reagents makes it possible to do quantitative proteomics and perform comparative protein expression measurements on very small samples.

In this chapter, Section 9.2 includes Molecular Probes’ reagents and kits (including the Quant-iT™, NanoOrange®, CBQCA and EZQ® reagents) for quantitating total protein in solution. Section 9.3 includes the important SYPRO® stains for detecting and quantitating total protein on gels and blots. Reagents for the qualitative and quantitative detection of phosphorylation, glycosylation and other post-translation modifications are described in Section 9.4 and include:

- Click-iT® reagents for detecting nascent protein synthesis, as well as post-translational modifications
- Pro-Q® Diamond phosphoprotein gel and blot stains—a breakthrough technology for selectively detecting phosphoproteins in gels and on blots
- Pro-Q® Emerald 300 and Pro-Q® Emerald 488 glycoprotein gel and blot stains—effective and easy-to-use stains for detecting periodate-oxidized glycoproteins in gels and on blots

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

## REFERENCES

9.2 Protein Quantitation in Solution

Several colorimetric methods have been described for quantitating proteins in solution, including the widely used Bradford, Lowry and BCA (bicinchoninic acid) assays (Table 9.1). Because they rely on absorption-based measurements, however, these methods are inherently limited in both sensitivity and effective range. We have developed four unique fluorometric methods for quantitating proteins in solution—the Quant-iT™ Protein Assay Kit (Q33210), the NanoOrange® Protein Quantitation Kit (N6666), the CBQCA Protein Quantitation Kit (C6667) and the EZQ® Protein Quantitation Kit (R33200)—that outperform these conventional methods (Table 9.1). We also offer the Qubit® fluorometer (Q32857), which is designed to work seamlessly with the Quant-iT™ Assay Kits, as well as several other fluorescent reagents useful for protein detection in solution.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Detection Wavelength(s) (nm)</th>
<th>Sensitivity and Effective Range</th>
<th>Mechanism of Action</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quant-iT™ protein quantitation assay (Q33210, Q33211, Q33212)</td>
<td>470/570</td>
<td>Quasi-linear from 0.5 to 4 µg in a 200 µL assay volume, with a sample volume of 1–20 µL</td>
<td>Binds to detergent coating on proteins and hydrophobic regions of proteins; the unbound dye is nonfluorescent</td>
<td>• Extremely fast and easy—just add sample to diluted dye and read fluorescence • High sensitivity • Little protein-to-protein variation • Compatible with salts, solvents, 2-mercaptoethanol, amino acids and DNA, but not detergents</td>
</tr>
<tr>
<td>NanoOrange® protein quantitation assay (N6666)</td>
<td>470/570</td>
<td>10 ng/mL to 10 µg/mL</td>
<td>Binds to detergent coating on proteins and hydrophobic regions of proteins; the unbound dye is nonfluorescent</td>
<td>• High sensitivity • Little protein-to-protein variation • Rapid assay with a simple procedure • Compatible with reducing agents, but not detergents</td>
</tr>
<tr>
<td>CBQCA protein quantitation assay (C6667)</td>
<td>450/550</td>
<td>10 ng/mL to 150 µg/mL</td>
<td>Reacts with primary amine groups on proteins in the presence of cyanide or thiols; the unreacted dye is nonfluorescent</td>
<td>• High sensitivity • Sensitivity depends on the number of amines present • Linear over an extended range of protein concentration • Compatible with detergents and lipophilic proteins • Not compatible with buffers containing amines or thiols</td>
</tr>
<tr>
<td>EZQ® protein quantitation assay (R33200)</td>
<td>280 and 450/618</td>
<td>50 µg/mL to 5 mg/mL, with a sample volume of 1 µL</td>
<td>Binds electrostatically to basic amino acids, supplemented by additional hydrophobic interactions</td>
<td>• Ideal for determining protein concentration prior to electrophoresis • Solid-phase format designed for high-throughput analysis • Little protein-to-protein variation • Compatible with detergents, reducing agents, urea and tracking dyes</td>
</tr>
</tbody>
</table>

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 | • 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 | Cu2+ is reduced to Cu+ in the presence of proteins at high pH; the BCA reagent chelates Cu+ ions, forming purple-colored complexes | • 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 | Cu2+ 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 | • Lengthy procedure with carefully timed steps • Not compatible with detergents or reducing agents |

Fluorescamine 4–7
(F2332, F20261) | 390/475 | 0.3 µg/mL to 13 µg/mL | Reacts with primary amine groups on proteins; unbound dye is nonfluorescent | • Sensitivity depends on the number of amines present • Reagent is unstable • Not compatible with amine-containing buffers |

OPA 8–10
(o-phthalaldehyde) (P2331MP) | 340/455 | 0.2 µg/mL to 25 µg/mL | Reacts with primary amine groups on proteins in the presence of 2-mercaptoethanol; unbound dye is nonfluorescent | • Sensitivity depends on the number of amines present • Not compatible with amine-containing buffers • Low cost |

UV absorption 11 | 280 | 10 µg/mL to 50 µg/mL or 50 µg/mL to 2 mg/mL | Peptide bond absorption; tryptophan and tyrosine absorption | • Sensitivity depends on the number of aromatic amino acid residues present • Nondestructive • Low cost |

* Excitation and emission wavelength maxima or absorption wavelength maximum, in nm.
Quant-iT™ Protein Assay Kits

Overview of the Quant-iT™ Assay Kits

The Quant-iT™ family of assay kits provides state-of-the-art reagents for sensitive and selective quantitation of protein, DNA or RNA (Table 8.8) samples using a standard fluorescence microplate reader (Figure 9.2.1) or the Qubit® fluorometer. These kits have been specially formulated with ready-to-use buffers, prediluted standards and easy-to-follow instructions, to help make quantitation both accurate and extremely easy. Each Quant-iT™ assay is:

- **Ready to use.** Only the dye is diluted in the supplied buffer; no dilution of standards or buffer required.
- **Easy to perform.** Just add the sample to the diluted dye and read the fluorescence.
- **Highly sensitive.** The Quant-iT™ assays are orders of magnitude more sensitive than UV absorbance measurements.
- **Highly selective.** Separate kits are available for quantitating protein, DNA or RNA (Section 8.3), with minimal interference from common contaminants.
- **Precise.** CVs are generally less than 5% for typical users.

The user-friendly Qubit® fluorometer has been designed to work seamlessly with the Quant-iT™ Assay Kits. The combination of the Quant-iT™ Assay Kits and the Qubit® fluorometer produces a completely integrated quantitation platform. Moreover, because the fluorescent dye in each Quant-iT™ Kit matches common fluorescence excitation and emission filter sets in microplate readers, these assay kits are ideal for high-throughput environments, as well as for small numbers of samples.

Quant-iT™ Protein Assay Kits

The Quant-iT™ Protein Assay Kit (Q33210) simplifies protein quantitation without sacrificing sensitivity. This protein assay exhibits a detection range between 0.25 and 5 µg protein, and the response curve is sigmoidal (quasi-linear from 0.5 to 4 µg) with little protein-to-protein difference in signal intensity (Figure 9.2.2). Common contaminants, including salts, solvents, reducing agents (dithiothreitol, 2-mercaptoethanol), amino acids, nucleotides and DNA, are well tolerated in this assay; however, it is not compatible with detergents; slight modifications in the procedure are required for other contaminants. Each Quant-iT™ Protein Assay Kit contains:

- Quant-iT™ protein reagent
- Quant-iT™ protein buffer
- A set of eight prediluted bovine serum albumin (BSA) standards between 0 and 500 ng/µL
- Easy-to-follow instructions

Sufficient reagents are provided to perform 1000 assays, based on a 200 µL assay volume in a 96-well microplate format; this assay can also be adapted for use in cuvettes or 384-well microplates. The fluorescence signal exhibits excitation/emission maxima of 470/570 nm and is stable for three hours at room temperature. The Quant-iT™ protein reagent is an improved formulation of Molecular Probes’ NanoOrange® reagent, which is described below.

We also offer Quant-iT™ Protein Assay Kits specifically designed for use with the Qubit® fluorometer (100-assay size, Q33211; 500-assay size, Q33212). Similar to the general Quant-iT™ Protein Assay Kit described above, these kits provide:

- Quant-iT™ protein reagent
- Quant-iT™ protein buffer
- Three pre-diluted protein standards
- Easy-to-follow instructions

The assay steps are simple: dilute the reagent using the buffer provided, add the sample (any volume between 1 µL and 20 µL is acceptable) and read the concentration using the Qubit® fluorometer. The assay is performed at room temperature, and the signal is stable for 3 hours. This assay is accurate for initial sample concentrations from 12.5 µg/mL to 5 mg/mL.
Qubit® Fluorometer

The Qubit® fluorometer (Figure 9.2.3) is designed to work seamlessly with the Quant-iT™ Assay Kits. Together, they form the Qubit® Quantitation Platform, an efficient combination of sophisticated, accurate, and highly sensitive fluorescence-based quantitation assays for DNA, RNA and protein, along with an extremely user-friendly fluorometer. This powerful pairing offers:

- Selective quantitation—more accurate than UV absorbance readings
- High sensitivity—use as little as 1 µL of sample for quantitation
- Intuitive integrated platform—sophisticated quantitation in 5 minutes or less

In addition to providing quantitation results that are fast, easy and reliable, the Qubit® fluorometer features software that is fully upgradable. The Qubit® fluorometer incorporates sophisticated data analysis algorithms designed to produce accurate measurements and an intuitive user interface for seamless integration with the full range of Quant-iT™ Assay Kits. We offer the Qubit® fluorometer separately (Q32857; USB cable, Q32858; replacement international power cord, Q32859) or bundled in a starter kit that includes four different Quant-iT™ assay kits as well as a supply of 500 Qubit® assay tubes (Q32860); a set of 500 Qubit® assay tubes is also available separately (Q32856).

Quant-iT™ Assay Kits use advanced Molecular Probes® fluorophores that become fluorescent upon binding to DNA, RNA or protein. The selectivity of these interactions ensures more accurate results than can be obtained with UV absorbance readings; Quant-iT™ Assay Kits only report the concentration of the molecule of interest, not sample contaminants. Quant-iT™ Assay Kits are up to 1000 times as sensitive as UV absorbance readings, and as little as 1 µL of sample is all that’s needed for accurate, reliable quantitation. We offer Quant-iT™ Assay Kits for a variety of quantitation needs:

- Quant-iT™ dsDNA Broad-Range (BR) and High-Sensitivity (HS) Assay Kits—for sequencing samples, genomic DNA samples and routine cloning experiments (Section 8.3)
- Quant-iT™ RNA Assay Kits—for microarray experiments, real-time PCR samples and northern blots (Section 8.3)
- Quant-iT™ Protein Assay Kits—for analysis of samples destined for western blotting and SDS-PAGE or 2D gel electrophoresis

More information is available at www.invitrogen.com/handbook/qubit.

NanoOrange® Protein Quantitation Kit

The NanoOrange® Protein Quantitation Kit (N6666) provides an ultrasensitive assay for measuring the concentration of proteins in solution.²,⁴ 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 9.2.4). Protein samples are simply added to the diluted NanoOrange® reagent in a lipid-containing medium, 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 the lipid-coated proteins with the NanoOrange® reagent produces a large fluorescence enhancement that can be used to generate a standard curve for protein determination; fluorescence of the reagent in aqueous solutions 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 9.2.5).
• **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 9.2.6). 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. 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

The NanoOrange protein quantitation reagent, with an excitation/emission maxima of 470/570 nm when bound to proteins, is suitable for use with a variety of instrumentation. Fluorescence is typically measured using instrument settings or filters that provide excitation/emission at ~485/590 nm, which are commonly available for both spectrophotometers and microplate readers. A spectrophotometer—either a standard fluorometer or a mini fluorometer—offers the greatest effective range and lowest detection limits for this assay. With fluorescence microplate 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 (N6666) supplies:

- Concentrated NanoOrange reagent in dimethylsulfoxide (DMSO)
- Concentrated NanoOrange diluent
- Bovine serum albumin (BSA) as a protein reference standard
- Detailed protocols 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 fluorometer or mini fluorometer, 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 electrophoresis and western blot analysis. 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. The NanoOrange reagent is also an optimal reagent for detecting proteins that have been separated by microchip capillary electrophoresis. A high-throughput assay that may be suitable for clinical samples has been developed for quantitating human serum albumin using a fluorescence microplate reader and using capillary electrophoresis laser-induced fluorescence (CE-LIF). 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 cultures and a rapid method for demonstrating flagellar movement of bacteria.12

![Figure 9.2.4](image-url) **Figure 9.2.4** Protein quantitation with the NanoOrange Protein Quantitation Kit. The NanoOrange assay (N6666) is simple to perform: after adding diluted dye, the samples are heated to denature the proteins, cooled to room temperature and the fluorescence read in either a microplate reader or a fluorometer.

![Figure 9.2.5](image-url) **Figure 9.2.5** Quantitative analysis of bovine serum albumin (BSA) using the NanoOrange Protein Quantitation Kit (N6666). 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 9.2.6](image-url) **Figure 9.2.6** Quantitative analysis of six different proteins using the NanoOrange Protein Quantitation Kit (N6666): A) bovine serum albumin (BSA), B) IgG, C) trypsin, D) carbonic anhydrase (CA), E) IgG, F) streptavidin (Stab) 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 using a microplate reader with excitation/emission wavelengths set at 485 ± 20 nm/590 ± 35 nm.
that the detection range can extend up to 150 µg. Inset B shows detection of BSA from 50 ng to 1000 ng. Inset A shows Figure 9.2.8

The primary plot point is the average of three determinations.

485 ± 10 nm and emission detection at 530 ± 12.5 nm. Each

using a fluorescence microplate reader with excitation at

of detergent (data not shown). Fluorescence was measured

X-100. Similar results were obtained without the addition

0.1 M sodium borate bu

then performed using the CBQCA Protein Quantitation Kit

Figure 9.2.9

Quantitation of proteins in a lipid environment using the CBQCA Protein Quantitation Kit (C6667). The protein concentrations of an LDL preparation and a bovine

Protein (ng)

Figure 9.2.10

Comparison of the fluorometric quantitation of bovine serum albumin (BSA) using ATTO-TAG™ CBQCA (which is supplied in the CBQCA Protein Quantitation Kit, C6667), OPA (P2331MP) or fluorescamine (F2332, F20261). BSA samples were derivatized using large molar excesses of the fluorogenic reagents and were analyzed using a fluorescence 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.

CBQCA Protein Quantitation Kit

The ATTO-TAG™ CBQCA reagent (A6222) was originally developed as a chromatographic derivatization reagent for amines13–15 (Section 1.8), but this reagent is also useful for quantitating proteins by virtue of its rapid and quantitative reaction with their accessible amines. We have developed the CBQCA Protein Quantitation Kit (C6667, Figure 9.2.7, Figure 9.2.8), which employs the ATTO-TAG™ CBQCA reagent for rapid and sensitive protein quantitation in solution12,16 (Table 9.1). The CBQCA protein quantitation assay functions well in the presence of lipids and detergents16,17 substances that interfere with many other protein determination methods.16 For example, the CBQCA-based assay can be used directly to determine the protein content of lipoprotein samples or lipid–protein mixtures (Figure 9.2.9). The CBQCA assay has been shown to give faster and more sensitive detection of both free amino acids in human plasma18 and both low and high molecular weight primary amines in clinical samples from hemo dialysis.19 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 9.2.10). 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 9.2.8). Alternatively, the reaction mixtures can be diluted to 1–2 mL for fluorescence measurement in a standard fluorometer or minifluorometer.

Each CBQCA Protein Quantitation Kit (C6667) contains:

- ATTO-TAG™ CBQCA detection reagent
- Potassium cyanide
- Dimethylsulfoxide (DMSO)
- Bovine serum albumin (BSA) protein reference standard
- Detailed protocols for protein quantitation

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

EZ® Protein Quantitation Kit

The EZ® Protein Quantitation Kit (R33200) provides a fast and easy high-throughput assay for proteins. Because detergents, reducing agents, urea and tracking dyes do not interfere, this fluorescence-based protein quantitation assay is ideal for determining the protein concentration of samples prior to polyacrylamide gel electrophoresis.20 This convenient kit can also provide a quick assessment of protein content during protein purification schemes and fractionation procedures.

The EZ® assay requires only 1 µL of a sample per spot, and up to 96 samples, including standards, can be assayed in one session. The protein samples are simply spotted onto one of the provided assay papers, fixed with methanol and then stained with the EZ® protein quantitation reagent. This assay paper is then clamped to the specially designed 96-well microplate.
which has only six lysine residues, will be detected less e
cessible primary amines.

Other Reagents for Protein Quantitation in Solution

Most traditional fluorogenic 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), 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, will be detected less efficiently than egg white avidin (MW ~66,000 daltons), which has 36 lysine residues, and bovine serum albumin (MW ~66,000 daltons), which has 59 lysine residues. However, the assays are generally rapid and easy to conduct, particularly in miniaturized and fluorescence microplate reader formats.

Certain dyes that detect primary aliphatic amines, including ATTO-TAG™ CBQCA (A6222), fluorescamine (F2332, F20261) and o-phthalaldehyde (OPA, P2331MP), 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 (NDA, N1138; Section 1.8), have frequently been used for amino acid analysis of hydrolyzed proteins.

Fluorescamine

Fluorescamine (F2332, F20261) is intrinsically nonfluorescent but reacts in milliseconds with primary aliphatic amines, including peptides and proteins, to yield a fluorescent derivatived. This amine-reactive reagent has been shown to be useful for determining protein concentrations of aqueous solutions and for measuring the number of accessible lysine residues in proteins. Protein quantitation with fluorescamine is particularly well suited to a minifluorometer or fluorescence microplate reader. Fluorescamine can also be used to detect proteins in gels and to analyze low molecular weight amines by TLC, HPLC and capillary electrophoresis.

Figure 9.2.13 Fluorogenic amine-derivatization reaction of fluorescamine (F2332, F20261).

Figure 9.2.12 Protein-to-protein variation in the EZQ™ protein quantitation assay. Triplicate 1 µg samples of various proteins were assayed using the EZQ™ Protein Quantitation Kit (R33200) and a fluorescence microplate reader. The mean fluorescence values, after correcting for background fluorescence, are expressed relative to that of ovalbumin. The coefficient of variation is ~16%. The protein samples are: A, ovalbumin; B, bovine serum albumin (BSA); C, myoglobin; D, soybean trypsin inhibitor; E, β-casein; f, carbonic anhydrase; G, transferrin; H, mouse IgG; I, lysozyme; and J, histones.

Figure 9.2.11 EZQ™ protein quantitation assay of ovalbumin. A dilution series of ovalbumin was prepared, assayed with the EZQ™ Protein Quantitation Kit (R33200) and then quantitated using both a 473 nm laser-based scanning instrument (top panel) and a fluorescence microplate reader (bottom panel). The assays were performed over a broad range; the insets show the low range in greater detail. The assays were performed in triplicate, and the mean values, in arbitrary fluorescence units, were plotted after subtracting background values of 86 (upper panel) or 18 (lower panel).

Each EZQ® Protein Quantitation Kit contains:

- EZQ® protein quantitation reagent
- EZQ® 96-well microplate cassette
- Assay paper
- Ovalbumin, for preparing protein standards
- Detailed protocols for protein quantitation using a variety of fluorescence-detection instruments

Sufficient reagent and assay paper are provided for ~2000 protein quantitation assays.
**o-Phthalaldehyde**

The combination of o-phthalaldehyde (OPA, P2331MP) and 2-mercaptoethanol provides a rapid and simple method of determining protein concentrations in the range of 0.2 µg/mL to 25 µg/mL. (Figure 9.2.14). As compared with 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. The OPA assay for lysine content is reasonably reliable over a broad range of proteins. OPA can also be used to detect increases in the concentration of free amines that result from protease-catalyzed protein hydrolysis.

**SYPRO® Red and SYPRO® Orange Protein Gel Stains**

An assay has been reported that uses the SYPRO® Red protein gel stain (S6653, S6654; Section 9.3) for quantitating total protein content of bacterial cells by flow cytometry. This assay provides an accurate method to measure planktonic bacterial biomass in marine samples. Fluorescence of the SYPRO® Orange protein gel stain (S6650, S6651; Section 9.3) has been used to follow isothermal protein denaturation (Note 9.1—Monitoring Protein-Folding Processes with Environment-Sensitive Dyes) and to selectively stain proteins in biofilms prior to two-photon laser-scanning microscopy.

**REFERENCES**


**DATA TABLE 9.2 PROTEIN QUANTITATION IN SOLUTION**

<table>
<thead>
<tr>
<th>Cat. No.</th>
<th>MW</th>
<th>Storage</th>
<th>Soluble</th>
<th>Abs</th>
<th>EC</th>
<th>Em</th>
<th>Solvent</th>
<th>Notes</th>
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<tbody>
<tr>
<td>A6222</td>
<td></td>
<td>F,D,L</td>
<td>MeOH</td>
<td>465</td>
<td>ND</td>
<td>560</td>
<td>MeOH</td>
<td>1, 2, 3</td>
</tr>
<tr>
<td>F2332</td>
<td></td>
<td>F,D,L</td>
<td>MeCN</td>
<td>380</td>
<td>7800</td>
<td>464</td>
<td>MeCN</td>
<td>4</td>
</tr>
<tr>
<td>F20261</td>
<td></td>
<td>F,D,L</td>
<td>MeCN</td>
<td>380</td>
<td>8400</td>
<td>464</td>
<td>MeCN</td>
<td>4, 5</td>
</tr>
<tr>
<td>F2331MP</td>
<td></td>
<td>L</td>
<td>EtOH</td>
<td>334</td>
<td>5700</td>
<td>455</td>
<td>pH 9</td>
<td>6</td>
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</table>

For definitions of the contents of this data table, see "Using The Molecular Probes® Handbook" in the introductory pages.

**PRODUCT LIST 9.2 PROTEIN QUANTITATION IN SOLUTION**

<table>
<thead>
<tr>
<th>Cat. No.</th>
<th>Product</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6222</td>
<td>ATTO-TAG™ CBQCA derivatization reagent (CBQCA; 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde)</td>
<td>10 mg</td>
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<tr>
<td>C5667</td>
<td>CBQCA Protein Quantitation Kit <em>300–800 assays</em></td>
<td>1 kit</td>
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<tr>
<td>F33200</td>
<td>EZQ® Protein Quantitation Kit <em>2000 assays</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>F2332</td>
<td>fluorescamine</td>
<td>100 mg</td>
</tr>
<tr>
<td>F20261</td>
<td>fluorescamine <em>FluoroPure™ grade</em></td>
<td>100 mg</td>
</tr>
<tr>
<td>N6666</td>
<td>NanoOrange® Protein Quantitation Kit <em>200–2000 assays</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>P2331MP</td>
<td>o-phthalaldehyde (OPA) <em>high purity</em></td>
<td>1 g</td>
</tr>
<tr>
<td>Q33211</td>
<td>Quant-IT™ Protein Assay Kit, 100 assays <em>0.25–5 µg</em> <em>for use with the Qubit® fluorometer</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>Q33212</td>
<td>Quant-IT™ Protein Assay Kit, 500 assays <em>0.25–5 µg</em> <em>for use with the Qubit® fluorometer</em></td>
<td>1 kit</td>
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<td>Q33210</td>
<td>Quant-IT™ Protein Assay Kit, 1000 assays <em>0.25–5 µg</em></td>
<td>1 kit</td>
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<tr>
<td>Q23656</td>
<td>Qubit® assay tubes <em>set of 500</em></td>
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</tr>
<tr>
<td>Q32857</td>
<td>Qubit® fluorometer</td>
<td>each</td>
</tr>
<tr>
<td>Q32859</td>
<td>Qubit® fluorometer international power cord <em>replacement</em></td>
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<td>Q32858</td>
<td>Qubit® fluorometer USB cable</td>
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</tr>
<tr>
<td>Q32860</td>
<td>Qubit® Quantitation Starter Kit</td>
<td>1 kit</td>
</tr>
</tbody>
</table>
9.3 Protein Detection on Gels, Blots and Arrays

SYPRO® Protein Gel Stains

The luminescent SYPRO® protein gel stains are revolutionizing the detection of the total-protein profile in polyacrylamide gels. SYPRO® protein gel stains exhibit several important characteristics that together make them far superior to traditional staining methods, including:

- Fast and easy staining protocols
- High sensitivity
- Minimal protein-to-protein variation in staining
- Broad linear quantitation range
- Compatibility with subsequent microanalysis and a variety of instrumentation

Conventional methods for universal profiling of proteins in gels include Coomassie brilliant blue staining and silver staining. Although Coomassie brilliant blue is an inexpensive reagent, its staining is relatively insensitive and time consuming. Silver staining may be up to 100 times more sensitive than Coomassie brilliant blue staining, but it is relatively expensive and entails several labor-intensive and time-sensitive steps. Silver staining also exhibits a high degree of protein-to-protein variability; staining intensity and color are very dependent on each polypeptide’s sequence and degree of glycosylation, and some proteins are detectable only as negatively stained patches. Moreover, silver staining shows very poor linearity with protein concentration (Figure 9.3.1) and poor reproducibility in staining from gel to gel, making it inadequate for comparative studies of protein expression in cells.

Figure 9.3.1 Quantitation of proteins in gels using SYPRO® Ruby protein gel stain versus silver stain. Dilutions of proteins were electrophoresed on eight different SDS-polyacrylamide gels, two gels for each of four dilution ranges. The gels were stained with either SYPRO® Ruby protein gel stain (S12000, S12001, S21900) or a silver stain. Staining intensities were quantitated using either the Fluor-S MAX Multimager documentation system (Bio-Rad® Laboratories) or the FLA3000G laser scanner (Fuji® Photo Film Co.) and plotted against the protein amount for bovine serum albumin. SYPRO® Ruby protein gel stain shows a linear quantitation range over three orders of magnitude, as well as consistent staining intensities from gel to gel. In contrast, the silver stain shows linear quantitation over only a small range, a very shallow slope and inconsistent staining intensities from gel to gel, even when corrected for background differences.
Many of the drawbacks of these traditional stains can be overcome by using one of the SYPRO® protein gel stains, without sacrificing detection sensitivity. We have developed a SYPRO® dye optimized for protein profiling in nearly every type of gel (Table 9.2) or blot application (Table 9.3). The characteristics and applications of the individual SYPRO® protein gel and blot stains for detecting the total-protein profile of a sample are described in this section. Section 9.4 discusses the use of these SYPRO® stains in conjunction with phosphoprotein and glycoprotein detection reagents for multiparameter staining.

**SYPRO® Ruby Protein Gel Stain: Ultrasensitive Protein Detection in 1D, 2D and IEF Gels**

SYPRO® Ruby protein gel stain (S12000, S12001, S21900) is a ready-to-use protein stain that has sensitivity equal to or exceeding that of the best silver staining techniques, is compatible with Edman sequencing of the individual SYPRO® protein gel and blot stains for detecting the total-protein profile of a sample. SYPRO® Ruby protein gel stain offers several advantages over conventional staining techniques:

- **High-sensitivity staining.** SYPRO® Ruby protein gel stain provides at least the same subnanogram sensitivity as the best silver staining techniques in 1D, 2D, or IEF gels (Figure 9.3.2).
- **Simple staining protocol.** After fixation, the gel is incubated in the staining solution. No stop solutions or destaining steps are required and, unlike silver staining, gels can be left in the dye solution for indefinite periods without overstaining, vastly simplifying the simultaneous processing of multiple gels and making it possible to perform high-throughput staining without investing in robotic staining devices.
- **Accurate peptide and protein detection.** SYPRO® Ruby protein gel stain shows little protein-to-protein variability in staining and detects some proteins that are completely missed by silver staining (Figure 9.3.3), such as heavily glycosylated proteins. Unlike silver staining, SYPRO® Ruby dye does not stain extraneous nucleic acids, lipids or carbohydrates in the sample.
- **Excellent performance in comparative protein expression studies.** SYPRO® Ruby stain shows a greater linear quantitation range than either silver or Coomassie brilliant blue staining—extending over three orders of magnitude—making it possible to accurately compare protein expression levels (Figure 9.3.1, Figure 9.3.2). Gel-to-gel staining is extremely consistent; same-spot intensity comparisons between identical 2D gels show a correlation coefficient of 0.9. Multiple gels can easily be compared using available software (Figure 9.3.4). Other protein quantitation methods, including running multiple prestained samples on the same gel, generally do not produce results that approach this level of discrimination.

### Table 9.2 SYPRO® and Coomassie Fluor™ luminescent and fluorescent protein gel stains.

<table>
<thead>
<tr>
<th>Dye (Cat. No.)</th>
<th>Ex/Em *</th>
<th>Major Applications</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYPRO® Ruby protein gel stain (S12000, S12001, S21900)</td>
<td>280, 450/610</td>
<td>2D gels, IEF gels, 1D SDS-PAGE</td>
<td>• High sensitivity (1–2 ng/band; comparable to silver staining) • Linear quantitation range over three orders of magnitude</td>
</tr>
<tr>
<td>SYPRO® Orange protein gel stain (S6650, S6651)</td>
<td>300, 470/570</td>
<td>1D SDS-PAGE</td>
<td>• Very good sensitivity (4–8 ng/band; higher than Coomassie brilliant blue staining) • Little protein-to-protein variability • Linear quantitation range over three orders of magnitude</td>
</tr>
<tr>
<td>SYPRO® Red protein gel stain (S6652, S6654)</td>
<td>300, 530/630</td>
<td>1D SDS-PAGE</td>
<td>• Very good sensitivity (4–8 ng/band; higher than Coomassie brilliant blue staining) • Linear quantitation range over three orders of magnitude</td>
</tr>
<tr>
<td>SYPRO® Tangerine protein gel stain (S12010)</td>
<td>300, 490/640</td>
<td>1D SDS-PAGE, blotting applications, zymography, electroelution</td>
<td>• Very good sensitivity (4–8 ng/band; higher than Coomassie brilliant blue staining) • Little protein-to-protein variability • Linear quantitation range over three orders of magnitude • No fixation required</td>
</tr>
<tr>
<td>Coomassie Fluor™ Orange protein gel stain (C33250, C33251)</td>
<td>300, 470/570</td>
<td>1D SDS-PAGE</td>
<td>• Premixed and ready-to-use solution • Very good sensitivity (8 ng/band; higher than Coomassie brilliant blue staining) • Little protein-to-protein variability • Linear quantitation range over at least two orders of magnitude</td>
</tr>
</tbody>
</table>

All SYPRO® gel and blot stains are compatible with Edman sequencing and mass spectrometry. *Excitation (Ex) and emission (Em) maxima, in nm. For maximum sensitivity, use excitation sources and optical filters matched to these values.

### Table 9.3 Molecular Probes® fluorescent and luminescent protein blot stains.

<table>
<thead>
<tr>
<th>Dye (Cat. No.)</th>
<th>Ex/Em *</th>
<th>Major Applications</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYPRO® Ruby protein blot stain (S11791)</td>
<td>280, 450/618</td>
<td>Mass spectrometry, microsequencing, counterstain for blot-based detection techniques (nitrocellulose or PVDF membranes)</td>
<td>• High sensitivity (1–2 ng/band; comparable to colloidal gold staining) • Reversible</td>
</tr>
<tr>
<td>BODIPY® FL-X succinimidyl ester (D6102) †</td>
<td>365, 505/575</td>
<td>Counterstain for blot-based detection techniques (PVDF membranes)</td>
<td>• Very good sensitivity (4–8 ng/band) • Permanent, covalent staining for multicolor techniques</td>
</tr>
<tr>
<td>BODIPY® TR-X succinimidyl ester (D6116) †</td>
<td>300, 590/615</td>
<td>Counterstain for blot-based detection techniques (PVDF membranes)</td>
<td>• Very good sensitivity (4–8 ng/band) • Permanent, covalent staining for multicolor techniques</td>
</tr>
</tbody>
</table>

* Excitation (Ex) and emission (Em) maxima, in nm. For maximum sensitivity, use excitation sources and optical filters matched to these values. † Available as a stand-alone reagent (D6102, Section 1.4).
Chapter 9 — Protein Detection and Analysis

Section 9.3  Protein Detection on Gels, Blots and Arrays

- **Compatibility with microsequencing and mass spectrometry.** Unlike silver staining techniques, which use glutaraldehyde- or formaldehyde-based fixatives, SYPRO® Ruby dye is a gentle stain that interacts noncovalently with proteins. Thus, high-quality Edman sequencing or mass spectrometry data (Figure 9.3.5) can be obtained immediately after staining, without modification steps that may compromise sensitivity. Automated in-gel digestion methods have been used in the analysis of femtomole levels of SYPRO® Ruby dye–stained proteins.

- **Utility for isoelectric focusing (IEF).** SYPRO® Ruby protein gel stain also provides a reliable, high-sensitivity staining method for isoelectric focusing (IEF) gels (Figure 9.3.6) without the problems typically encountered with silver staining, such as ampholyte staining or mirroring effects on the plastic gel backing.

- **Minimal hazardous waste.** As compared with silver stains, SYPRO® Ruby protein gel stain generates much less hazardous waste, minimizing the time and expense associated with waste disposal.

![Figure 9.3.2](image)

**Figure 9.3.2** Amounts of carbonic anhydrase ranging from 1 ng to 1000 ng were separated on an SDS-polyacrylamide gel and stained with SYPRO® Ruby protein gel stain (S12000, S12001, S21900). The inset shows the excellent linearity in the lower part of the range from 1 ng to 60 ng protein. Staining intensities were quantitated using the Molecular Imager® FX documentation system (Bio-Rad® Laboratories). For comparison, the gray band shows the linear range for the same protein detected with silver staining.

![Figure 9.3.3](image)

**Figure 9.3.3** SYPRO® Ruby protein gel stain (S12000, S12001, S21900) shows less protein-to-protein variation than silver staining. Proteins from a cell lysate were run on a 2D gel and stained with SYPRO® Ruby protein gel stain (left) or silver stain (right). The grayscale values of the SYPRO® Ruby dye–stained gel have been inverted for easier comparison with the silver-stained gel.

![Figure 9.3.4](image)

**Figure 9.3.4** Comparison of two protein samples run on 2D gels. Proteins from either a normal liver tissue sample or a liver tumor sample were run on two 2D gels and stained with SYPRO® Ruby protein gel stain (S12000, S12001, S21900). Images of the gels were captured using the FLA-3000 scanner (Fuji®). Images from a portion of the two gels were then pseudocolored either pink or green, overlaid and matched spot-for-spot using Z3 software (Compugen). Green spots represent proteins expressed in the liver tumor samples; pink spots represent proteins expressed in the normal liver tissue sample. Black spots represent proteins expressed in both tissues.

![Figure 9.3.5](image)

**Figure 9.3.5** Mass spectrum profile of NADH:ubiquinone reductase precursor (75,000-dalton subunit) obtained after 2D gel electrophoresis of bovine heart mitochondria and staining with SYPRO® Ruby protein gel stain (S12000, S12001, S21900). Bovine heart mitochondria were a gift of Roderick Capaldi, University of Oregon.

![Figure 9.3.6](image)

**Figure 9.3.6** SYPRO® Ruby protein gel stain versus silver stain for IEF gels. Serial dilutions of isoelectric focusing protein standards were electrophoresed on two identical polyacrylamide gels. One gel was stained with SYPRO® Ruby protein gel stain (S12000, S12001, S21900) (left) and the other with silver stain (right). Both stains show a similar limit of sensitivity for all proteins.
SYPRO® Ruby protein gel stain is based on an organometallic ruthenium complex, which shows an extremely bright and photostable red-orange luminescence when excited with either UV or blue light (Figure 9.3.7). Stained proteins can be visualized using a UV transilluminator, a blue-light transilluminator or a laser-scanning instrument. Gels can then be documented using Polaroid® 667 black-and-white print film, a CCD camera with an image documentation system or a laser-scanning instrument. For optimal sensitivity using a UV transilluminator and Polaroid® 667 black-and-white print film, the SYPRO® photographic filter (S6656) is recommended.

SYPRO® Ruby protein gel stain is supplied as 200 mL of a 1X staining solution (S12001), sufficient for staining about four minigels, or 1 L of a 1X staining solution (S12000), sufficient for staining about 20 minigels or two standard 2D gels. Additionally, we offer SYPRO® Ruby protein gel stain in a 5 L box (S21900), sufficient for staining about 100 minigels or 10 standard 2D gels. These boxes are easy to stack and store, and the convenient spigot makes it easy to dispense just the right amount of stain.

SYPRO® Orange and SYPRO® Red Protein Gel Stains: For Routine Detection of Proteins in 1D SDS-Polyacrylamide Gels

SYPRO® Orange (S6650, S6651) and SYPRO® Red (S6653, S6654) protein gel stains provide a fluorescence-based alternative for protein detection in SDS-polyacrylamide gels that is not only faster and more sensitive than Coomassie brilliant blue staining, but can be as sensitive as short-protocol silver staining methods (Figure 9.3.8) at a fraction of the time, effort and cost.23–28

In the presence of excess SDS, nonpolar regions of polypeptides are coated with detergent molecules, forming a micelle-like structure with a nearly constant SDS/protein ratio (1.4 g SDS:1.0 g protein); this constant charge-per-mass ratio is the basis of molecular weight determination by SDS-polyacrylamide gel electrophoresis. SYPRO® Orange and SYPRO® Red dyes bind to the SDS coat that surrounds proteins in SDS-polyacrylamide gels. Thus, the staining observed with these dyes exhibits relatively little protein-to-protein variation and is linearly related to protein mass (Figure 9.3.9). Some important features of SYPRO® Orange and SYPRO® Red protein gel stains include:

- **Ease of use.** Following electrophoresis, the gel is stained for 10–60 minutes and then briefly rinsed—no separate fixation, stop or destaining steps are required. After staining, the gel is immediately ready for photography, or it can be stored, in or out of the staining solution, for days.

**Figure 9.3.7** Luminescence excitation (dashed line) and emission (solid line) spectra of the SYPRO® Ruby protein gel stain (S12000, S12001, S21900) and SYPRO® Ruby protein blot stain (S11791).

**Figure 9.3.8** Comparison of the sensitivity achieved with SYPRO® Orange, SYPRO® Red, silver and Coomassie brilliant blue stains. Identical SDS-polyacrylamide gels were stained with A) SYPRO® Orange protein gel stain (S6650, S6651), B) SYPRO® Red protein gel stain (S6653, S6654), C) silver stain and D) Coomassie brilliant blue stain, according to standard protocols. SYPRO® Orange and SYPRO® Red dye–stained gels were photographed using 300 nm transillumination, a SYPRO® photographic filter (S6656) and Polaroid® 667 black-and-white print film. The silver- and Coomassie brilliant blue-stained gels were photographed with transmitted white light and Polaroid® 667 black-and-white print film; no photographic filter was used to photograph these gels.

**Figure 9.3.9** Quantitation of proteins in a gel using SYPRO® Orange protein gel stain (S6650, S6651). A protein mixture was serially diluted and electrophoresed on a 15% SDS-polyacrylamide gel and then stained with SYPRO® Orange protein gel stain. The gel was then scanned using a Storm gel and blot analysis system (excitation/emission 488/>520 nm) and analyzed to yield the fluorescence intensity of the stained bands. The fluorescence intensity scale is the same in both panels, illustrating the minimal protein-to-protein staining variation of SYPRO® Orange and SYPRO® Red dyes.

Fluorescence

![Fluorescence intensity](image)

**Figure 9.3.8** Comparison of the sensitivity achieved with SYPRO® Orange, SYPRO® Red, silver and Coomassie brilliant blue stains. Identical SDS-polyacrylamide gels were stained with A) SYPRO® Orange protein gel stain (S6650, S6651), B) SYPRO® Red protein gel stain (S6653, S6654), C) silver stain and D) Coomassie brilliant blue stain, according to standard protocols. SYPRO® Orange and SYPRO® Red dye–stained gels were photographed using 300 nm transillumination, a SYPRO® photographic filter (S6656) and Polaroid® 667 black-and-white print film. The silver- and Coomassie brilliant blue-stained gels were photographed with transmitted white light and Polaroid® 667 black-and-white print film; no photographic filter was used to photograph these gels.

**Figure 9.3.9** Quantitation of proteins in a gel using SYPRO® Orange protein gel stain (S6650, S6651). A protein mixture was serially diluted and electrophoresed on a 15% SDS-polyacrylamide gel and then stained with SYPRO® Orange protein gel stain. The gel was then scanned using a Storm gel and blot analysis system (excitation/emission 488/>520 nm) and analyzed to yield the fluorescence intensity of the stained bands. The fluorescence intensity scale is the same in both panels, illustrating the minimal protein-to-protein staining variation of SYPRO® Orange and SYPRO® Red dyes.

Fluorescence

![Fluorescence intensity](image)
- **High sensitivity.** SYPRO® Orange and SYPRO® Red protein gel stains routinely provide a sensitivity level of at least 8–16 ng per band in SDS-polyacrylamide minigels when visualized with standard 300 nm transillumination (Figure 9.3.8). Photography using Polaroid® 667 black-and-white print film and a SYPRO® photographic filter (S6656) enhances the detection of staining with SYPRO® Orange or SYPRO® Red dye by several-fold over visible detection because the film integrates the signal throughout the duration of the exposure. Laser scanners also detect nanogram quantities of SYPRO® dye–stained proteins in gels.

- **Broad linear quantitation range.** Protein detection in gels stained with either SYPRO® Orange or SYPRO® Red stain is linear over three orders of magnitude in protein quantity (Figure 9.3.9).

- **Uniform protein staining.** Unlike silver staining, SYPRO® Orange and SYPRO® Red dyes exhibit relatively low protein-to-protein variability in SDS-polyacrylamide gels (Figure 9.3.9) and do not stain nucleic acids, which are sometimes found in protein mixtures from cell or tissue extracts. In addition, SYPRO® Orange and SYPRO® Red dyes only weakly stain lipopolysaccharides in bacterial lysates, whereas these biopolymers are strongly detected by some types of silver staining. Glycoproteins (such as the IgG variable subunit) and proteins with prosthetic groups (such as bovine cytochrome oxidase) are also efficiently stained with SYPRO® Orange and SYPRO® Red dyes.

- **Photostability.** Proteins stained with SYPRO® Orange or SYPRO® Red dye are relatively photostable, enabling the researcher to acquire multiple photographic images and to use long film-exposure times (2–8 seconds). Gels that are illuminated for long periods of time may partially photobleach but can be restained with little loss of sensitivity.

- **Compatibility with many types of instruments.** Although their excitation maxima are in the visible range (Figure 9.3.10), SYPRO® dye–stained gels are readily visualized using standard 300 nm transilluminators. SYPRO® Orange protein gel stain also exhibits good sensitivity when viewed with a blue-light transilluminator, and both SYPRO® Orange and SYPRO® Red protein gel stains are suitable for use with many laser-scanning instruments.

- **Chemical stability.** SYPRO® Orange and SYPRO® Red gel stains are chemically stable; if protected from light, fluorescence of the stained gel is stable for several days, and staining solutions can be stored for months.

- **Economy.** SYPRO® Orange and SYPRO® Red gel stains are not only less expensive than silver-staining kits but faster and less laborious to use. Additionally, use of SYPRO® Orange or SYPRO® Red dye avoids the costs of purchase and disposal of organic solvents that are required for Coomassie brilliant blue staining.

- **Compatibility with mass spectroscopy.** Unlike silver stains, SYPRO® Orange and SYPRO® Red dyes do not covalently bind to proteins, allowing subsequent analysis of stained proteins by microsequencing or mass spectrometry.

SYPRO® Orange and SYPRO® Red stains have very similar staining properties, although we have observed that proteins stained with SYPRO® Orange dye are slightly brighter, whereas gels stained with SYPRO® Red dye tend to have lower background fluorescence. For maximum sensitivity with UV transilluminators, we recommend documenting the signal using Polaroid® 667 black-and-white print film and the SYPRO® photographic filter (S6656). For maximum sensitivity with laser scanners, we recommend matching the appropriate SYPRO® dye with the excitation light source of the instrument. SYPRO® Orange protein gel stain is most suitable for gel scanners that employ argon-ion lasers with output at 488 nm, whereas SYPRO® Red protein gel stain is best matched to laser-scanning instruments that employ Nd:YAG lasers with output at 532 nm. SYPRO® Red protein gel stain has been used as a prestain for protein analysis in an automated ultrathin-layer gel electrophoretic technique. SYPRO® Orange protein gel stain has been used for protein sizing on a microchip and for analyzing the kinetics of isothermal protein denaturation (Note 9.1—Monitoring Protein-Folding Processes with Environment-Sensitive Dyes).

SYPRO® Orange and SYPRO® Red protein gel stains are compatible with SDS or urea/SDS gels. Staining native proteins in gels in the absence of SDS results in more protein-to-protein variation and lower sensitivity than staining SDS-denatured proteins, due to variations in...
with SYPRO® Orange protein gel stain (S6650, S6651). Separated on a 15% SDS-polyacrylamide gel and stained with SYPRO® Orange protein gel stain (S12000, S12001, S21900).

Because SYPRO® Orange and SYPRO® Red dyes do not covalently bind to proteins, stained proteins can be subsequently analyzed by microsequencing or mass spectrometry.¹¹,¹²,¹³ These dyes, however, are not recommended for staining gels prior to blotting, as there is a significant loss of sensitivity when proteins are stained with SYPRO® Orange or SYPRO® Red dyes in typical western blotting buffers. To obtain maximum sensitivity when staining proteins in western blotting protocols, we recommend SYPRO® Tangerine protein gel stain (S12010) for staining proteins on the gel before blotting or SYPRO® Ruby protein blot stain (S11791) for staining proteins on nitrocellulose or PVDF membranes after blotting.

SYPRO® Orange and SYPRO® Red protein gel stains are available as 500 µL stock solutions in dimethylsulfoxide (DMSO), either in a single vial (S6650, S6653) or specially packaged as a set of 10 vials, each containing 50 µL (S6651, S6654). The reagents are supplied as 5000X concentrates; thus, 500 µL of either stain yields 2.5 L of staining solution. Photography of proteins in gels, which is essential for obtaining the maximum sensitivity, requires use of the SYPRO® Tangerine protein gel stain.

SYPRO® Tangerine Protein Gel Stain: Sensitive Protein Detection without Fixation for Electroelution, Zymography and Classroom Use

SYPRO® Tangerine protein gel stain (S12010), which stains proteins in gels without the need for either acids or organic solvents, serves as an alternative to conventional protein stains that fix proteins in the gel.¹⁵ Whereas SYPRO® Orange and SYPRO® Red stains require a staining solution containing acetic acid for maximum performance, staining with SYPRO® Tangerine protein gel stain is possible in almost any buffer that contains NaCl. Because proteins are not fixed during the staining procedure, they can be readily eluted from gels, used for zymography (in-gel enzyme activity assays, Figure 9.3.11) or analyzed by mass spectrometry.¹⁶ SYPRO® Tangerine stain can also be used to stain gels before transferring the proteins to nitrocellulose or PVDF membranes for immunostaining (western blotting). Like SYPRO® Orange and SYPRO® Red protein gel stains, SYPRO® Tangerine protein gel stain shows high sensitivity (down to ~4 ng/band) and a broad linear quantitation range (Figure 9.3.12). Easy to use, SYPRO® Tangerine protein gel stain is also ideal for use in educational settings, especially when used with UV-free blue-light transilluminators.

SYPRO® Tangerine stain is compatible with conventional SDS-polyacrylamide gel electrophoresis, but it is not recommended for 2D or IEF gels. Stained proteins can be visualized using a UV transilluminator, a blue-light transilluminator or a laser scanner. Photography of stained gels using Polaroid® 667 black-and-white print film requires use of the SYPRO® photographic filter (S6656) for optimal sensitivity. SYPRO® Tangerine protein gel stain (S12010) is available as 500 µL of a 5000X concentration in dimethylsulfoxide (DMSO), an amount sufficient to stain about 100 minigels.

SYPRO® Protein Gel Stain Starter Kit

SYPRO® Orange, SYPRO® Red and SYPRO® Tangerine protein gel stains are all available in a SYPRO® Protein Gel Stain Starter Kit (S12012) for first-time users. Each kit includes:

- 50 µL of SYPRO® Orange protein gel stain, sufficient for 5–20 minigels
- 50 µL of SYPRO® Red protein gel stain, sufficient for 5–20 minigels
- 50 µL of SYPRO® Tangerine protein gel stain, sufficient for 5–20 minigels
- SYPRO® protein gel stain photographic filter
- Detailed protocols

Protein Molecular Weight Standards

We offer a protein mixture for use as molecular weight markers in SDS-polyacrylamide gel electrophoresis (Figure 9.3.13). This broad-range marker mixture (P6649) contains a balanced formulation of 11 polypeptides with molecular weights from 6500 to 205,000 daltons. These protein molecular weight standards give rise to sharp, well-separated bands when the gel is stained with any of our protein gel or blot stains. The mixture provides amounts of marker...
proteins sufficient for loading about 200 gel lanes. We also have available PeppermintStick™ phosphoprotein molecular weight standards (P33350) and CandyCane™ glycoprotein molecular weight standards (C21852), which are described in Section 9.4.

**Electrophoretic Mobility-Shift (Bandshift) Assay (EMSA) Kit**

To make bandshift assays easier, we offer the Electrophoretic Mobility-Shift Assay (EMSA) Kit (E33075), which provides a fast and quantitative fluorescence-based method for detecting both nucleic acid and protein in the same gel (Figure 9.3.14). This kit uses two different stains for detection: the fluorescent SYBR® Green EMSA nucleic acid gel stain for RNA or DNA and the luminescent SYPRO® Ruby EMSA protein gel stain for proteins. Because the nucleic acids and proteins are stained in the gel after electrophoresis, there is no need to prelabel the DNA or RNA with a radioisotope, biotin or a fluorescent dye before the binding reaction, and therefore there is no possibility that the label will interfere with protein binding. Staining takes only about 20 minutes for the nucleic acid stain, and about 4 hours for the subsequent protein stain, yielding results much faster than radioisotope labeling (which may require multiple exposure times) or chemiluminescence-based detection (which requires blotting and multiple incubation steps).

This kit also makes it possible to perform ratiometric measurements of nucleic acid and protein in the same band, providing more detailed information on the binding interaction. The signals from the two stains are linear over a broad range, allowing accurate determination of the amount of nucleic acid and protein, even in a single band, with detection limits of ~1 ng for nucleic acids and ~20 ng for protein. Both stains can be visualized using a standard 300 nm UV transilluminator, a 254 nm epi-illuminator or a laser scanner (Figure 9.3.14). For optimal sensitivity using a UV transilluminator and Polaroid® 667 black-and-white print film, the SYPRO® photographic filter (S6656) and the SYBR® photographic filter (S7569, Section 8.4) are recommended. Digital images can easily be overlaid for a two-color representation of nucleic acid and protein in the gel.

The EMSA Kit contains sufficient reagents for 10 nondenaturing polyacrylamide minigel assays, including:

- SYBR® Green EMSA nucleic acid gel stain
- SYPRO® Ruby EMSA protein gel stain
- Trichloroacetic acid, for preparing the working solution of SYPRO® Ruby EMSA protein gel stain
- Concentrated EMSA gel-loading solution
- Concentrated binding buffer
- Detailed protocols

**SYPRO® Photographic Filter**

To achieve optimal sensitivity with the SYPRO® stains, it is essential to photograph the gel or blot because the camera’s integrating capability can make bands visible that are not detected by eye. Photographs should be taken using a photographic filter with spectral properties closely matched to those of the fluorescent or luminescent dye used.

When using Polaroid® 667 black-and-white print film and UV illumination, protein gels or blots stained with any of the SYPRO® protein stains (including SYPRO® Ruby, SYPRO® Orange, SYPRO® Red and SYPRO® Tangerine gel stains as well as SYPRO® Ruby protein blot stain) should be photographed through the SYPRO® photographic filter (Figure 9.3.15, Figure 9.3.16), a 75 mm × 75 mm gelatin filter. Note that these gelatin filters are generally not suitable for use with portable or stationary gel-documentation systems or with CCD cameras.
1,8-ANS and bis-ANS (A47, B153; Section 13.5) have proven to be sensitive probes for partially folded intermediates in protein-folding pathways. These applications take advantage of the strong fluorescence enhancement exhibited by these amphiphilic dyes when their exposure to water is lowered (Figure 1, Figure 2). Consequently, fluorescence of ANS increases substantially when proteins to which it is bound undergo transitions from unfolded to fully or partially folded states that provide shielding from water. Molten globule intermediates are characterized by particularly high ANS fluorescence intensities due to the exposure of hydrophobic core regions that are inaccessible to the dye in the native structure. Binding of 1,8-ANS and bis-ANS to proteins is noncovalent and involves a combination of electrostatic and hydrophobic modes. Some investigators have noted that the dye-binding event itself may induce protein conformational changes, indicating the advisability of correlating ANS fluorescence measurements with data obtained using other physical techniques. In particular, high-resolution structural analysis of an ANS-protein complex by X-ray crystallography has demonstrated the occurrence of local rearrangements of the protein structure to accommodate the dye.

The general mechanism of protein-folding detection by ANS has been developed as the basis of fluorescence thermal shift (a.k.a. differential scanning fluorimetry) assays for high-throughput analysis of protein stability. The assay readout is a profile of protein-dye complex fluorescence intensity as a function of temperature. Profiles are obtained and compared for multiple samples in which environmental or structural factors influencing protein stability are systematically varied. Thermocycler instruments designed for real-time PCR monitoring provide a readily adaptable instrument platform for these measurements. Most high-throughput fluorescence thermal shift assays use SYPRO Orange dye (S6650, S6651; Section 9.3) instead of ANS. Other environment-sensitive dyes with demonstrated utility include SYPRO Red dye (S6653, S6654; Section 9.3), Dapoxyl sulfonylic acid (D12800, Section 13.5), nile red (N1142, Section 13.5) and CPM (D346, Section 2.3). Within the broad context of protein stability optimization, fluorescence thermal shift assays have many applications including:

- Analysis of ligand binding to proteins of unknown function
- Identification of protein–protein interaction inhibitors
- Analysis of protein stabilization by peptide aptamers and amino acid ligands
- Characterization of engineered protein variants
- Optimization of protein crystallization conditions
- Enhancement of recombinant protein quality and yield

Coomassie Fluor™ Orange Protein Gel Stain

Coomassie Fluor™ Orange protein gel stain (C33250, C33251) provides a fast, simple and sensitive method for staining of proteins in SDS-polyacrylamide electrophoretic gels. Gels do not need to be washed before staining with the Coomassie Fluor™ Orange dye or destained after staining. After electrophoresis, the gel is simply stained, rinsed and photographed on a standard UV transilluminator. Our premixed and ready-to-use Coomassie Fluor™ Orange protein gel stain offers the following advantages over conventional colorimetric stains:

- **High sensitivity.** Coomassie Fluor™ Orange protein gel stain detects as little as 8 ng of protein per minigel band (Figure 9.3.17). This sensitivity matches the best colloidal Coomassie stains and exceeds standard Coomassie brilliant blue stains.
- **Broad linear range of detection.** The fluorescence intensity of Coomassie Fluor™ Orange dye–stained bands is linear with protein quantity over at least two orders of magnitude, permitting accurate quantitation.
- **Rapid staining.** Staining is complete in less than an hour, and there is no risk of overstaining the gel.
- **Compatibility with standard laboratory equipment.** Stained proteins can be visualized using a standard 300 nm UV transilluminator or a laser scanner (Figure 9.3.18). For optimal sensitivity using a UV transilluminator and Polaroid® 667 black-and-white print film, the SYPRO® photographic filter (S6656) is recommended.
- **Low protein-to-protein variability.** Because Coomassie Fluor™ Orange dye interacts with the SDS coat around proteins in the gel, it gives more consistent staining between different types of proteins, as compared with Coomassie brilliant blue staining, and it never exhibits negative staining. Coomassie Fluor™ Orange dye also stains glycoproteins.
- **High selectivity for proteins.** Coomassie Fluor™ Orange protein gel stain detects a variety of proteins down to ~6500 daltons without staining nucleic acid or lipopolysaccharide contaminants that are sometimes found in protein preparations derived from cell or tissue extracts.

Coomassie Fluor™ Orange protein gel stain is not recommended for staining proteins in 2D, IEF or nondenaturing gels; for these applications we recommend our SYPRO® Ruby protein gel stain (S12000, S12001, S21900).

Rhinohide™ Polyacrylamide Gel Strengthener

Rhinohide™ polyacrylamide gel strengthener improves upon classic polyacrylamide gel technology by making gels much stronger, providing easier handling and much greater resistance to tearing without adverse side effects (Figure 9.3.19). Rhinohide™ polyacrylamide gel strengthener is highly recommended for low-percentage gels, large-format gels and gels subject to multiple staining and handling steps, and it is compatible with fluorescent protein stains as well as silver and Coomassie stains.

SDS-polyacrylamide gels supplemented with Rhinohide™ polyacrylamide gel strengthener exhibit resolution capabilities comparable to traditional SDS-polyacrylamide gels, with clear, focused bands and without the undesirable side effects common with other gel strengtheners. For example, film-backed gels and polyester fabric–reinforced gels interfere with blotting techniques and can negatively affect protein staining. Alternatively, strengthening gels by the addition of pre-formed polymers causes turbidity and can produce serious spot-morphology artifacts, such as the distortion of high molecular weight bands or doubling of protein spots in the molecular weight dimension of 2D gels.18 Rhinohide™ polyacrylamide gel strengthener produces gels with excellent transparency, providing exceptional image viewing and scanning of fluorescently stained gels with minimal background staining. We offer a concentrated form of the Rhinohide™ polyacrylamide gel strengthener (R33400) for adding to existing stock solutions of acrylamide/bis-acrylamide (37:5:1). Because prestained proteins, such as prestained molecular weight markers, will not migrate correctly in acrylamide gels containing the Rhinohide™ polyacrylamide gel strengthener, we recommend using only unstained proteins as markers.
**SYPRO® Ruby Protein Blot Stain**

To characterize specific proteins in complex mixtures, proteins are frequently separated by electrophoresis, then blotted onto nitrocellulose or PVDF (poly(vinylidene difluoride)) membranes (blots) for immunostaining (western blotting), glycoprotein staining, sequencing or mass spectrometry. Total-protein stains provide valuable information about the protein samples on blots, making it possible to assess the efficiency of protein transfer to the blot, detect contaminating proteins in the sample and compare the sample with molecular weight standards. For blots of 2D gels, staining of the entire protein profile makes it easier to localize a protein to a particular spot in the complex protein pattern. The superior properties of our fluorescent and luminescent protein stains, compared with conventional colorimetric stains, make it possible to quickly and easily obtain this information without running duplicate gels. Our luminescent and fluorescent protein staining technology can also be combined with fluorescent reagents for glycoprotein and phosphoprotein detection, which are described in detail in Section 9.4, to create multiparameter staining for multiplex analyses.

SYPRO® Ruby protein blot stain \(^{3,16,39,40}\) (Table 9.3) is designed to provide fast and highly sensitive detection of proteins blotted onto membranes, making it easy to assess the efficiency of protein transfer to the blot and to determine if lanes are loaded equally \(^{41}\) (Figure 9.3.20). Because the stain does not covalently alter the proteins, it can be used to locate proteins on blots before sequencing or mass spectrometry.\(^ {4,39}\) It can also be used before chromogenic, fluorogenic or chemiluminescent immunostaining procedures to locate molecular weight markers and visualize the total-protein profile in the sample.\(^ {39}\) Furthermore, the stain does not interfere with subsequent identification techniques, minimizing the need to run duplicate gels, vastly simplifying the comparison of total protein and target protein on western blots, and allowing precise localization of the target protein relative to other proteins on electroblots of 2D gels. SYPRO® Ruby protein blot stain is also compatible with our Pro-Q® Emerald glycoprotein blot stains for glycoproteins \(^ {42}\) (Section 9.4). The superior properties of SYPRO® Ruby protein blot stain, as compared with conventional protein blot stains, make it possible to routinely stain blots for total protein before continuing with specific protein detection techniques. SYPRO® Ruby protein blot stain \(^ {3,16,39,40}\) (S11791) combines the following superior staining characteristics:

- **High sensitivity.** SYPRO® Ruby protein blot stain can detect as little as 0.25–1 ng protein/mm\(^2\) (~2–8 ng/band) blotted onto PVDF or nitrocellulose membranes after only 15 minutes of staining \(^ {39}\) (Figure 9.3.20). This sensitivity on blots is far better than that of colorimetric stains, such as Ponceau S, amido black or Coomassie brilliant blue, and rivals the best colloidal gold blot-staining techniques (Figure 9.3.21).
- **Rapid total-protein staining procedure.** The SYPRO® Ruby protein blot-staining protocol takes less than an hour—including fixation and wash steps—and maximum sensitivity is achieved after only 15 minutes of dye staining, even for some peptides as small as seven amino acids. In contrast, gold or silver staining procedures may require overnight incubations to achieve maximum sensitivity and usually include extensive wash procedures that must be carefully timed.

- **Compatibility with western blot protocols.** Staining the total-protein profile on the blot minimizes guesswork about transfer efficiency and removes the need to run two gels for comparison of total and target protein. SYPRO® Ruby protein blot stain is gentle and, unlike colorimetric or colloidal gold blot stains, does not interfere with subsequent colorimetric or chemiluminescent immunodetection of proteins on western blots.

- **Compatibility with standard microsequencing and mass spectrometry protocols.** Whereas colloidal gold, Coomassie brilliant blue and amido black staining can interfere with post-staining analysis, SYPRO® Ruby protein blot stain binds noncovalently to proteins and is thus fully compatible with Edman sequencing or mass spectrometry.

SYPRO® Ruby protein blot stain is based on an organometallic ruthenium complex. Because the ruthenium complex has dual-excitation maxima (Figure 9.3.7), the dye exhibits luminescence upon excitation with either UV or visible light. This property makes it possible to visualize the luminescence with many types of instruments, including UV epi-illumination sources, UV or blue-light transilluminators and a variety of laser-scanning instruments, including those with excitation light at 450 nm, 473 nm, 488 nm or 532 nm. Also, SYPRO® Ruby dye–stained proteins can be indirectly visualized by the chemiluminescence of the high-energy intermediate produced in the reaction of bis-(2,4,6-trichlorophenyl) oxalate (TCPO) with H₂O₂. The red luminescence of the ruthenium complex has a peak at ~618 nm that is well separated from these excitation peaks, minimizing the amount of background signal seen from the excitation source. The staining signal can be documented using Polaroid® 667 black-and-white print film and a SYPRO® photographic filter (S6656; Figure 9.3.15), using a CCD-based imaging system equipped with a 600 nm bandpass or 490 nm longpass filter, or by using the appropriate filter set and software in a laser scanner. SYPRO® Ruby protein blot stain has exceptional photostability, allowing long exposure times for maximum sensitivity.

SYPRO® Ruby protein blot stain (S11791) is supplied as a 1X staining solution, which is sufficient for staining ~1600 cm² of blotting membrane, and is accompanied by a detailed protocol for its use.

### Protein Detection on Microarrays

**SYPRO® Ruby Protein Blot Stain for Reversible Protein Detection on Microarrays**

We have found that SYPRO® Ruby protein blot stain (S11791) performs particularly well when staining proteins on PVDF microarrays for quality control and normalization purposes. SYPRO® Ruby protein blot stain shows good sensitivity on protein microarrays (Figure 9.3.22) and should be very useful for staining proteins before exposing the microarray to the sample. The stain washes off PVDF membranes very easily under conditions used with typical western blot blocking buffers.

**Reactive Fluorescent Dyes for Permanent Protein Detection on Microarrays**

The BODIPY® reactive dyes described in Section 1.4 label amine groups (predominantly lysine residues) on proteins. As compared to the reversible SYPRO® blot stains described above, the amine-reactive BODIPY® FL-X succinimidyl ester (D6102) shows even greater sensitivity for microarray staining (Figure 9.3.22), making it useful for quality control testing or as an internal normalization standard. Both BODIPY® FL-X succinimidyl ester and the red-fluorescent BODIPY® TR-X succinimidyl ester (D6116) are particularly effective general stains for proteins on PVDF membranes (Table 9.3).

This unique method of staining total protein on blots with the reactive BODIPY® dyes has an approximately 30-fold linear dynamic range (Figure 9.3.23), although the absolute intensity between proteins may vary somewhat with the nature of the protein. Reactive BODIPY® dye–based staining is designed to be rapid, simple and highly sensitive, permitting detection of as little as 4 ng of a protein per band in about an hour. Because the reactive dyes form a...
Protein Detection in Capillary Electrophoresis

Capillary electrophoresis (CE) is an exceptionally powerful tool for the resolution of biomolecules. Fluorescent detection of proteins that are separated by capillary electrophoresis can occur either during the run—the more common procedure—or subsequent to the separation on isolated fractions. When detected during the electrophoretic separation, the protein is either derivatized with a fluorescent reagent prior to the separation or labeled with a fluorescent dye that is incorporated into the running medium. In general, the same reagents may be useful for fluorometric detection of peptides and proteins that are separated by high-performance liquid chromatography (HPLC). In addition to the total-protein staining techniques described below, many selective staining techniques, such as the use of BODIPY® FL GTP-γ-S (G22183, Section 17.3) to detect GTP-binding proteins, can be applied to proteins separated by capillary electrophoresis.

Use of SYPRO® Dyes for Capillary Electrophoresis

SDS-capillary gel electrophoresis (SDS-CGE) separates proteins based on principles similar to those of standard slab-gel electrophoresis, but with the advantages of faster run times, higher resolution and greater sensitivity. The use of online detection by laser-induced fluorescence (LIF) increases the sensitivity several orders of magnitude over UV detection, eliminates the time spent staining and photographing the gel and allows for the possibility of automated sample processing. SDS-CGE analyzed by LIF is widely used for the separation and identification of DNA fragments and has increased the efficiency of genomics, DNA typing and forensics laboratories. SDS-CGE promises to be just as useful for proteomics laboratories and other laboratories that require characterization of a large number of protein samples.

For SDS-CGE of protein samples, the amine groups of lysine residues and the N-terminus of the proteins are typically derivatized with a fluorescent or fluorogenic dye such as the ATTO-TAG™ CBQCA (A2333, A6222) or ATTO-TAG™ FQ (A2334, A10192) reagents before separation in the capillary. The derivatized proteins are then coated with SDS and travel through the capillary gel towards the positive electrode based on their size, with smaller proteins traveling faster. The derivatized proteins are detected by fluorescence emitted as they pass a laser that excites the fluorophores.

One disadvantage of this labeling technique is that the proteins generally contain multiple amine groups, each of which can react with the derivatization reagent. Typically, only a few of the amine groups on each protein molecule react, and the result is an enormous number of different derivatives, creating broad peaks that may be difficult to correlate with the original protein’s structure or abundance. In addition, variations between runs make it difficult to reproducibly estimate molecular weights. In contrast, use of SYPRO® Red protein gel stain (S6653, S6654) to precast SDS-coated proteins allows more accurate determination of molecular weights because the proteins are relatively uniformly coated with SDS and the dye. Staining the SDS-coated proteins with SYPRO® Red protein gel stain leads to molecular weight determinations similar in accuracy to those achieved with polyacrylamide slab gels, with a limit of detection estimated to exceed the detection limit of silver staining in slab gels.

The NanoOrange® reagent (N6666, Section 9.2) is also reported to be an effective reagent for detecting proteins that have been separated by microchip capillary electrophoresis.
Protein Derivatization Reagents

Several of the same reagents that were described in Section 9.2 for protein quantitation in solution are also useful for peptide and protein derivatization, either prior to or following separation by capillary electrophoresis. However, chemical derivatization prior to separation is likely to change the electronic charge and always changes the mass of the protein. Furthermore, incomplete derivatization of amines or thiols on the protein can lead to a pure protein resolving into multiple species in the electrophoretogram.

In an improved procedure for fluorescent analysis of peptides by capillary electrophoresis, Zhou and colleagues 60 modified all ε- and ε- amino groups of the peptide with phenyl isothiocyanate. Following one cycle of Edman degradation, the single free ε- amino group was modified with fluorescent reagents to give a homogeneous, dye-labeled peptide.

The preferred reagents for derivatizing amine residues in proteins either prior to or following electrophoretic separation are those that are essentially nonfluorescent until reacted with the protein. These include:

- ATTO-TAG™ CBQCA, which is available in the ATTO-TAG™ CBQCA Amine-Derivatization Kit (A2333) and as a stand-alone reagent (A6222). ATTO-TAG™ CBQCA reacts with primary amines to form highly fluorescent isocyanates 50,61–69 and has been extensively used for the derivatization of amino acids, 51,61,70–72 peptides, 63,73–76 and carbohydrates 62,64,67,77–81 prior to capillary electrophoretic separation. ATTO-TAG™ CBQCA has been used to derivatize a fusion protein expressed in the bacterium Escherichia coli before purification by capillary zone electrophoresis. After purification, the fluorescent isocyanide can be removed by acid treatment to allow sequencing of the purified protein. 50
- ATTO-TAG™ FQ (3-(2-furoyl)quinoline-2-carboxaldehyde), which is available in our ATTO-TAG™ FQ Amine-Derivatization Kit (A2334) and as a stand-alone reagent (A10192). ATTO-TAG™ FQ has been used as a protein detection reagent in capillary electrophoresis. 82–85 It has been reported that ATTO-TAG™ FQ can detect as little as 200 attomoles of a protein by capillary electrophoresis. 82–85 Excitation of amine derivatives of ATTO-TAG™ FQ by the 488 nm spectral line of the argon-ion laser is more efficient than that of ATTO-TAG™ CBQCA derivatives. A report 85 describes the solid-phase derivatization of dilute peptide solutions (10−8 M) that have been immobilized on Immobilon® CD membranes. This technique permits the quantitative derivatization and analysis by capillary electrophoresis of only a few picomoles of the analyte.
- Fluorescamine (F2332, F20261), a nonfluorescent reagent that rapidly reacts with amines to give a fluorescent product. Fluorescamine has been used for solution quantitation of proteins and peptides (Section 9.2). It is also useful as a peptide and protein detection reagent for capillary electrophoresis. 76,86,87 Use of fluorescamine to derivatize a standard protein of known molecular weight together with use of the ATTO-TAG™ FQ reagent to derivatize the sample protein allows the sample to be run simultaneously with the standard, improving the accuracy of molecular weight determination. 83 Chiral separation of fluorescamine-labeled amino acids has been optimized using capillary electrophoresis in the presence of hydroxypropyl-β-cyclodextran, a method designed for use in extraterrestrial exploration on Mars. 88
- Dithiothreitol (DTT) and N-ethylmaleimide (NEM) have also been used to detect thiol-containing proteins in SDS gels and by reverse-phase HPLC, 50 and 5-iodoacetamidofluorescein (I30451, Section 2.2) has been used to label proteins for analysis by capillary electrophoresis. 101

Chapter 1 describes a variety of other amine-reactive reagents that have been used or may be useful for peptide and protein detection in capillary electrophoresis, including dansyl chloride 97 (D21), NBD chloride (C20260), NBD fluoride (F486), FITC 60,98 (F143), Pacific Blue™ succinimidyl ester (P10163) 99 and other common reagents described in Section 1.8.

Derivatization reagents that react with thiols or other functional groups have also been used for protein detection in capillary electrophoresis. Thiol-reactive reagents that are essentially nonfluorescent until conjugated to thiols, such as the coumarin maleimides CPM and DACM (D346, D10251), monobromobimane (M1378, M20381) and N-(1-pyrene)maleimide (P28), should work well in this application and are described in Section 2.3. Although intrinsically fluorescent, BODIPY® iodoacetamides and maleimides (Section 2.2) have also been used to detect thiol-containing proteins in SDS gels and by reverse-phase HPLC, 50 and 5-iodoacetamidofluorescein (I30451, Section 2.2) has been used to label proteins for analysis by capillary electrophoresis. 101

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REFERENCES


DATA TABLE 9.3  PROTEIN DETECTION ON GELS, BLOTS AND ARRAYS

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<td>see Notes</td>
<td>L</td>
<td>see Notes</td>
<td>462</td>
<td>see Notes</td>
<td>610</td>
<td>MeOH</td>
<td>13, 14, 15</td>
</tr>
<tr>
<td>S12001</td>
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<td>L</td>
<td>see Notes</td>
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<td>D,L</td>
<td>DMSO</td>
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<td>H₂O/BSA</td>
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<tr>
<td>S21900</td>
<td>see Notes</td>
<td>L</td>
<td>see Notes</td>
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<td>see Notes</td>
<td>610</td>
<td>MeOH</td>
<td>13, 14, 15</td>
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For definitions of the contents of this data table, see “Using The Molecular Probes® Handbook” in the introductory pages.

Notes
1. 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−1 M−1), nonfluorescent.
2. ND is not determined.
3. Solubility in methanol is improved by addition of base (e.g., 1–5% (v/v) 0.2 M KOH).
4. Data represent the reactive dye component of this labeling kit.
5. Spectral data are for the reaction product with glycine in the presence of cyanide. Unreacted reagent in MeOH: Abs = 282 nm (EC = 21,000 cm−1 M−1), nonfluorescent.
6. Fluorescine spectra are for the reaction product with butylamine. The fluorescence quantum yield and lifetime of the butylamine adduct in EtOH are 0.23 and 7.5 nanoseconds, respectively.
7. Product is specified to equal or exceed 98% analytical purity by HPLC.
8. Spectral data are for the reaction product with glycine in the presence of cyanide, measured in pH 7.0 buffer/MeCN (40:60). (Anal Chem (1987) 59:1102) Unreacted reagent in MeOH: Abs = 279 nm (EC = 5500 cm−1 M−1), Em = 330 nm.
9. Spectral data are for the reaction product of P2331MP with alamine and 2-mercaptoethanol. The spectra and stability of the adduct depend on the amine and thiol reactants. (Biochim Biophys Acta (1979) 576:440) Unreacted reagent in H₂O: Abs = 257 nm (EC = 1000 cm−1 M−1).
10. This product is supplied as a ready-made solution in the solvent indicated under “Soluble.”
11. The active ingredient of this product is an organic dye with MW <1000. The exact MW and extinction coefficient values for this dye are proprietary.
12. Abs and Em values are for the dye complexed with bovine serum albumin (H₂O/BSA).
13. This product is supplied as a ready-made aqueous staining solution.
14. The active ingredient of this product is an organometallic complex with MW <1500. The exact MW value and extinction coefficient of the complex are proprietary.
15. SYPRO® Ruby protein gel stain also has an absorption peak at 278 nm with about 4-fold higher EC than the 462 nm peak.
## PRODUCT LIST 9.3 PROTEIN DETECTION ON GELS, BLOTS AND ARRAYS

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<td>A2333</td>
<td>ATTO-TAG™ CBQCA Amine-Derivatization Kit</td>
<td>1 kit</td>
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<tr>
<td>A6222</td>
<td>ATTO-TAG™ CBQCA derivatization reagent (CBQCA; 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde)</td>
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<tr>
<td>A2334</td>
<td>ATTO-TAG™ FQ Amine-Derivatization Kit</td>
<td>1 kit</td>
</tr>
<tr>
<td>A10192</td>
<td>ATTO-TAG™ FQ derivatization reagent (FQ; 3-(2-furoyl)quinoline-2-carboxaldehyde)</td>
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<td>C33250</td>
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<tr>
<td>D6116</td>
<td>6-(((4-(4,4-di(fluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenox)-acetyl)amino)hexanoic acid, succinimidyl ester (BODIPY® TR-X, SE)</td>
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<td>E33075</td>
<td>Electrophoretic Mobility-Shift Assay (EMSA) Kit <em>with SYBR® Green and SYPRO® Ruby EMSA stains</em> <em>10 minigel assays</em></td>
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<td>S6650</td>
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<tr>
<td>S6656</td>
<td>SYPRO® photographic filter</td>
<td>each</td>
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<tr>
<td>S6654</td>
<td>SYPRO® Red protein gel stain <em>5000X concentrate in DMSO</em> <em>special packaging</em></td>
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<td>S11791</td>
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<td>SYPRO® Ruby protein gel stain</td>
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<td>SYPRO® Ruby protein gel stain</td>
<td>1 L</td>
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<td>SYPRO® Ruby protein gel stain <em>bulk packaging</em></td>
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<td>S12010</td>
<td>SYPRO® Tangerine protein gel stain <em>5000X concentrate in DMSO</em></td>
<td>500 µL</td>
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9.4 Detecting Protein Modifications

Click-iT® Reagents for Detecting Protein Synthesis and Modifications

Click-iT® labeling technology employs a bioorthogonal reactive chemistry for the in situ labeling of specific molecular populations, such as proteins that have been newly synthesized or post-translationally modified in some experimental time window of interest. The Click-iT® labeling reaction is based on a copper-catalyzed azide–alkyne cycloaddition \(^{12}\) and derives its high degree of specificity from the fact that the azide and alkyne reaction partners have no endogenous representation in biological molecules, cells, tissues or model organisms.\(^3\)–\(^5\)

Application of this reaction to in situ labeling of cells is a two-step process. First, one reaction partner—either an azide or alkyne linked to a “building block” such as an amino acid, monosaccharide, fatty acid, nucleotide or nucleoside—is biosynthetically incorporated. Subsequently, the other reaction partner—the complementary alkyne or azide linked to a fluorescent dye, biotin or other detection reagent—is “clicked” into place in the presence of catalytic copper (I), providing a detection moiety (Figure 9.4.1). One reaction partner must be an azide derivative and the other an alkyne derivative, but either functional moiety can serve as the biosynthetically incorporated molecule or the detection molecule\(^6\) (e.g., L-azidohomoalanine (AHA) + Alexa Fluor\(^g\) 488 alkyne is the inverse of the reaction scheme shown in Figure 9.4.1A).

The small size of alkyne and azide tags allows the biosynthetic building blocks to which they are attached to be processed by enzymes, such as aminocyl tRNA synthetases and nucleotide polymerases, that have poor tolerance for substrates with larger modifications such as fluorescent organic dyes.\(^7\) Furthermore, the 1,2,3-triazole linkage between the azide and alkyne reaction partners (Figure 9.4.1) is extremely stable. It is not susceptible to hydrolysis, oxidation or reduction, and it survives ionization in mass spectrometry (MS) analysis. Click-iT® labeling technology and the details of the click reaction are discussed in Section 3.1. For a complete list of azide and alkyne derivatives compatible with Click-iT® labeling technology, see Table 3.1. Here we highlight the azide and alkyne derivatives that can be used for labeling newly synthesized proteins and detecting post-translational protein modifications.

Azide- and Alkyne-Modified Amino Acids

The Click-iT® AHA (L-azidohomoalanine, C10102) and Click-iT® HPG (L-homopropargylglycine, C10186; Figure 9.4.1) reagents are methionine surrogates that provide nonradioactive alternatives to \(^{35}\)S-methionine for pulse-chase detection of protein synthesis and degradation.\(^8\)–\(^11\) These amino acid analogs are fed to cultured cells and incorporated into proteins during active protein synthesis. The enzymatically incorporated Click-iT® AHA or Click-iT® HPG is then detected with a fluorescent alkyne or fluorescent azide, respectively, using a Cu(I)-catalyzed click reaction. These Click-iT® reagents provide detection sensitivity comparable to that obtained using the radioactive \(^{35}\)-methionine method and are compatible with downstream LC-MS/MS and MALDI MS analysis, as well as with total-protein, glycoprotein and phosphoprotein gel stains for differential analyses of newly synthesized protein together with post-translational modifications.

Click-iT® AHA is also available in the Click-iT® AHA Alexa Fluor\(^g\) 488 Protein Synthesis HCS Assay Kit (C10289), which provides Alexa Fluor\(^g\) 488 alkyne for detection. Click-iT® AHA has proven to be a successful substitute for methionine in many cell types, including COS-7,

![Figure 9.4.1](image-url) 

Figure 9.4.1 Click-iT® copper-catalyzed azide–alkyne cycloaddition chemistry applied to detection of A) proteins and B) carbohydrates. The reaction partners are A) L-homopropargylglycine (HPG) and Alexa Fluor\(^g\) 488 alkyne and B) azidocetylgalactosamine and Alexa Fluor\(^g\) 488 alkyne. In both cases, the left-hand partner is a metabolic precursor that can be incorporated into proteins via de novo synthesis or post-translational modification pathways.
3T3-L1, HeLa, HEK 293 and Jurkat cells. Note that cells should be labeled in methionine-free media, as methionine is the preferred substrate for methionyl tRNA transferase, and supplemented media (i.e., methionine-free DMEM) should be used in place of HBSS to achieve greater Click-iT™ AHA incorporation at lower concentrations.

Azide- and Alkyne-Modified Monosaccharides

The Click-iT™ metabolic glycoprotein labeling reagents provide biosynthetic precursors for detecting and characterizing post-translational glycosylation of proteins. Four azide- or alkyne-modified monosaccharides are available for metabolic incorporation into a specific subclass of protein glycan structures:

- Click-iT™ GalNAz metabolic glycoprotein labeling reagent (tetraacetylated N-azidoacetylglactosamine, C33365; Figure 9.4.1), for labeling O-linked glycoproteins
- Click-iT™ ManNAz metabolic glycoprotein labeling reagent (tetraacetylated N-azidoacetylmannosamine, C33366; Figure 9.4.2), for labeling sialic acid–modified glycoproteins
- Click-iT™ GlcNAz metabolic glycoprotein labeling reagent (tetraacetylated N-azidoacetylgalactosamine, C33367; Figure 9.4.3), for labeling O-linked N-acetylglucosamine (O-GlcNAc)–modified glycoproteins
- Click-iT™ fucose alkyne (tetraacetylfucose alkyne, C10264; Figure 9.4.4), for labeling fucosylated proteins

Cultured cells are simply incubated with the modified sugars for 2–3 days or until cells reach the appropriate density. The acetyl groups improve cell permeability of the modified sugars and are removed by nonspecific intracellular esterases (Figure 9.4.2). The resulting azide- or alkyne-modified sugar is then metabolically incorporated through the permissive nature of the oligosaccharide biosynthesis pathway, yielding functionalized glycoproteins that can be chemoselectively coupled to complementary alkyne- or azide-functionalized fluorophores and biotinylation reagents for detection or affinity capture. We offer three Click-iT™ Protein Analysis Detection Kits (C33370, C33371, C33372) described below for the detection of azide-functionalized glycoproteins in 1D or 2D electrophoresis gels or western blots. These labeled glycoproteins are compatible with total-protein, glycoprotein and phosphoprotein gel stains and provide a detection sensitivity of a few hundred femtomoles, allowing an in-depth analysis of low-abundance glycoproteins as well as glycoproteins with a small degree of glycosylation.

Glycoproteins labeled with the Click-iT™ labeling and detection reagents are also compatible with downstream LC-MS/MS and MALDI-MS analyses for further identification and characterization. For added convenience, we offer an O-GlcNAc peptide LC/MS standard (C33374) from the transcription factor CREB for LC-MS/MS and MALDI-MS analyses of the O-GlcNAc posttranslational modification. This peptide is also available together with its phosphorylated counterpart for use as LC/MS standards (C33373) in differential mass spectrometry–based studies of the corresponding modifications, as well as for characterizing differential β-elimination/addition conditions.

Figure 9.4.2 Metabolic incorporation of tetraacetylated azido sugars.

Figure 9.4.3 Click-iT™ GlcNAz metabolic glycoprotein labeling reagent (tetraacetylated N-azidoacetylgalactosamine for labeling O-linked N-acetylglucosamine O-GlcNAc–modified glycoproteins; C33367).

Figure 9.4.4 Click-iT™ fucose alkyne (tetraacetylfucose alkyne) (C10264).
Click-iT® O-GlcNAc Enzymatic Labeling System

We also offer the the Click-iT® O-GlcNAc Enzymatic Labeling System for in vitro enzyme-mediated N-azoacetylglucosaminyl labeling of O-GlcNAc–modified glycoproteins. Proteins are enzymatically labeled using the permissive mutant β-1,4-galactosyltransferase (Gal-T1, Y289L), which transfers azido-modified galactose (GalNAz) from UDP-GalNAz to O-GlcNAc residues on the target proteins. Target proteins can then be detected using an alkyne-derivatized fluorophore or one of the Click-iT® Protein Analysis Detection Kits described below. Using the Click-iT® O-GlcNAc Enzymatic Labeling System in conjunction with the Click-iT® Tetrathioethane (TAMRA) Protein Analysis Detection Kit, we have detected as little as 1 picomole of α-crystallin, a protein which is only 2–10% O-GlcNAc modified.

Each Click-iT® O-GlcNAc Enzymatic Labeling System provides:

- UDP-GalNAz
- Gal-T1 (Y289L)
- Click-iT® O-GlcNAc enzymatic labeling buffer
- MnCl₂
- α-crystallin, for use as a positive control
- Detailed labeling protocols

Azide-Modified Isoprenoids and Fatty Acids

We offer several azide-modified isoprenoids and fatty acids, including:

- Click-iT® farnesyl alcohol, azide (C10248), for identifying farnesylated proteins
- Click-iT® geranylgeranyl alcohol, azide (C10249), for identifying geranylgeranylated proteins
- Click-iT® palmitic acid, azide (15-azidopentadecanoic acid, C10265), for identifying protein fatty acylation
- Click-iT® myristic acid, azide (12-azidododecanoic acid, C10268), for identifying protein fatty acylation

These azide-functionalized isoprenoids and fatty acids enable detection of post-translational lipidation of proteins by in-gel fluorescence scanning, fluorescence microscopy and flow cytometry.¹⁶⁻²⁰

Azide- and Alkyne-Derivatized Dyes and Biotinylation Reagents

We offer a rich selection of azide- and alkyne-derivatized fluorescent dyes for coupling to complementary azide- and alkyne-functionalized biomolecules (Table 3.1, Section 3.1), including:

- Alexa Fluor® 488 azide (A10266, Figure 9.4.1) and alkyne (A10267, Figure 9.4.1)
- Alexa Fluor® 555 azide (A20013) and alkyne (A10275)
- Alexa Fluor® 647 azide (A10277) and alkyne (A10278)
- Oregon Green® 488 azide (O10180) and alkyne (O10181)
- Tetramethylrhodamine (TAMRA) azide (T10182) and alkyne (T10183)
- Biotin azide (B10184) and alkyne (B10185)

Antibodies to Oregon Green® 488, tetramethylrhodamine and Alexa Fluor® 488 dyes (Section 7.4) and Tyramide Signal Amplification (TSA™) Kits (Section 6.2) are available to provide signal amplification if necessary. The biotin azide and alkyne reagents facilitate western blotting applications and streptavidin enrichment in combination with our streptavidin and CaptAvidin™ agarose matrices (S951, C21386; Section 7.6).

Click-iT® Protein Analysis Detection Kits

In addition to azide- and alkyne-derivatized dyes and biotinylation reagents, we offer three Click-iT® Protein Analysis Detection Kits (C33370, C33371, C33372) that provide labeled alkynes for the detection of azide-labeled biomolecules. These Click-iT® Protein Analysis Detection Kits provide sufficient reagents for 10 labeling reactions based on the provided protocol and include:

- TAMRA alkyne (in C33370), Dapoxy® alkyn (in C33371) or biotin alkyne (in C33372)
- Click-iT® reaction buffer
- CuSO₄
- Click-iT® reaction buffer additives
- Detailed labeling protocols

Click-iT® Reaction Buffers

For added convenience, we offer Click-iT® Reaction Buffer Kits for protein or cell samples labeled with an azide- or alkyne-tagged biomolecule. The Click-iT® Cell Reaction Buffer Kit (C10269) includes sufficient reagents to perform 50 reactions based on a 0.5 mL reaction volume for subsequent analyses by flow cytometry, fluorescence microscopy or high-content screening (HCS). The Click-iT® Protein Reaction Buffer Kit (C10276) includes everything required for click coupling to functionalized proteins for subsequent standard protein biochemical analyses (e.g., western blots or mass spectrometry).

Pro-Q® Diamond Phosphoprotein Stain for Gels and Blots

We have developed a suite of compatible methodologies for the differential staining of specific proteins (phosphoproteins, glycoproteins or membrane proteins) and the total-protein profile in two or more visually distinguishable colors, producing a more complete picture of the proteome. This set of protein stains not only offer the capacity for the simultaneous detection of multiple protein targets in a single sample, but also provides a combination of high sensitivity and simplicity that can streamline protocols in 1D and 2D polyacrylamide gels or on blots.

The Pro-Q® Diamond phosphoprotein gel stain, Pro-Q® Emerald glycoprotein gel stains and SYPRO® Ruby protein gel stain—which we have optimized to complement each other in selectivity, sensitivity and staining protocols—can be used in serial detection of phosphoproteins, glycoproteins and total proteins on a single protein sample separated by 1D or 2D gel electrophoresis (Figure 9.4.5). Our Rhinohide™ polyacrylamide gel strengtheners (R33400, Section 9.3) greatly improves the strength of any polyacrylamide gel, making it...
easy to perform these multiple staining procedures without special gel handling. After each staining step, an image of the gel is collected. Once collected, the three images can be overlaid in any combination for analysis of phosphorylation, glycosylation and total-protein expression. Because all three stains are used on the same gel, unambiguous spot matching of phosphoproteins and glycoproteins is made simple by direct comparison with the total-protein profile provided by the SYPRO® Ruby protein gel stain. This simultaneous measurement of several variables allows for perfect spatial registration of signals and increases the amount of data that can be collected in a single experiment, leading to more controlled experiments, more accurate data comparisons and fewer ambiguities.

Pro-Q® Diamond Phosphoprotein Gel Stain and Destain

Pro-Q® Diamond phosphoprotein gel stain is a breakthrough technology that provides a simple, direct method for selectively staining O-linked phosphoproteins in polyacrylamide gels (Figure 9.4.6). It is ideal for the identification of kinase targets in signal transduction pathways and for phosphoproteomic studies. This proprietary fluorescent stain allows direct, in-gel detection of phosphate groups attached to tyrosine, serine or threonine residues. The Pro-Q® Diamond phosphoprotein gel stain can be used with standard SDS-polyacrylamide gels.

Figure 9.4.6 Selectivity of the Pro-Q® Diamond phosphoprotein gel stain (P33300, P33301, P33302) for phosphoproteins. A polyacrylamide gel containing various proteins was stained with A) Pro-Q® Diamond phosphoprotein gel stain, followed by B) SYPRO® Ruby protein gel stain. This gel shows a nonphosphorylated protein, lysozyme (lanes 3 and 4), as well as several phosphoproteins, α-casein (lanes 1 and 2), ovalbumin (lanes 5 and 6) and pepsin (lanes 7 and 8), before (even lanes) and after (odd lanes) treatment with phosphatases. Loss of Pro-Q® Diamond staining indicates loss of all phosphates from pepsin, partial loss of phosphates from α-casein and ovalbumin and no change in the nonphosphorylated protein lysozyme.

Figure 9.4.5 An overview of the Multiplexed Proteomics® approach. Images collected after each staining step can be overlaid in any combination for analysis of protein expression, phosphorylation and glycosylation between samples.
gels (Figure 9.4.6) or with 2D gels (Figure 9.4.7)—blotting is not required and there is no need for radioisotopes, phosphoprotein-specific antibodies or western blot detection reagents. The simple and reliable staining protocol delivers results in as little as 4 to 5 hours. The stain is also compatible with mass spectrometry, allowing analysis of the phosphorylation state of entire proteomes. The Pro-Q® Diamond phosphoprotein gel stain provides:

- **Simple in-gel detection.** Proteins containing phosphate groups attached to tyrosine, serine or threonine residues can be detected directly in either 1D or 2D polyacrylamide gels after the gel is fixed, stained and destained; no antibodies are required and no blotting is necessary.

- **Selectivity without radioactivity.** The Pro-Q® Diamond phosphoprotein gel stain is a fluorescent stain that selectively detects phosphoproteins; radioisotopes are not used and therefore no radioactive waste is generated.

- **Sensitivity.** The Pro-Q® Diamond phosphoprotein gel stain allows the detection of as little as 1–16 ng of phosphoprotein per band, depending on the phosphorylation level of the protein.

![Figure 9.4.7 Visualization of total protein and phosphoproteins in a 2D gel. Proteins from a Jurkat T-cell lymphoma line cell lysate were separated by 2D gel electrophoresis and stained with Pro-Q® Diamond phosphoprotein gel stain (P33300, P33301, P33302, blue) followed by SYPRO® Ruby protein gel stain (S12000, S12001, S21900, red). After each dye staining, the gel was imaged on an FLA-3000 scanner (Fujifilm®). The digital images were acquired using Z3 software (Compugen), and the resulting composite image was digitally pseudocolored and overlaid.](image)

![Figure 9.4.9 Fluorescence excitation and emission spectra of the Pro-Q® Diamond phosphoprotein gel stain (P33300, P33301, P33302).](image)

![Figure 9.4.8 Sensitivity and linear range of Pro-Q® Diamond phosphoprotein gel stain (P33300, P33301, P33302). Six different proteins were serially diluted and run on separate SDS-polyacrylamide gels, which were then stained with Pro-Q® Diamond phosphoprotein gel stain. The images were documented on a fluorescence imager, and the fluorescence emission from each band was quantitated. The number of known phosphate groups on each protein is indicated in the figure legend. A) Fluorescence emission of the band, plotted as a function of protein amount, in nanograms. B) Magnification of data points in the highlighted box in panel A. C) The fluorescence emission of the band, plotted as a function of picomoles of protein. D) Magnification of the data points in the highlighted box in panel C. E) The slope of the line for each protein in panel C, plotted against the known number of phosphates per protein.](image)
• **Quantitation.** The Pro-Q® Diamond signal for individual phosphoproteins is linear over three orders of magnitude and correlates with the number of phosphate groups (Figure 9.4.8).

• **Compatibility.** Pro-Q® Diamond gel stain (excitation/emission maxima ~555/580 nm; Figure 9.4.9) is compatible with a visible-light–scanning instrument, a visible-light transilluminator or (with reduced sensitivity) a 300 nm transilluminator, as well as with mass spectrometry analysis.

• **Multiplexing capability.** Pro-Q® Diamond gel stain can be used with SYPRO® Ruby protein gel stain (Figure 9.4.6) and Pro-Q® Emerald glycoprotein gel stain on the same gel for multicolor gel staining.

The Pro-Q® Diamond phosphoprotein gel stain (Table 9.4) is supplied ready-to-use in three different sizes: a 200 mL size (P33301) suitable for staining approximately four minigels; a 1 L size (P33300) suitable for staining approximately 20 minigels or two large-format gels, e.g., 2D gels; and a 5 L bulk-packaging size (P33302). In addition, we offer Pro-Q® Diamond Phosphoprotein Gel Staining Kits (MPP33300, MPP33301, MPP33302) that include both the Pro-Q® Diamond gel stain and the PeppermintStick™ phosphoprotein molecular weight standards. All products are accompanied by a simple and reliable staining and destaining protocol that delivers results in as little as four to five hours. For convenient destaining, we also offer the Pro-Q® Diamond phosphoprotein gel destaining solution as a ready-to-use solution in either a 1 L (P33310) or 5 L (P33311) size.

**Multiplexed Proteomics® Kits for Phosphoprotein and Total-Protein Gel Staining**

When used together, the Pro-Q® Diamond phosphoprotein gel stain and the SYPRO® Ruby protein gel stain (S12000, S12001, S21900; Section 9.3) make a powerful combination for proteome analysis. The SYPRO® Ruby dye is a total-protein stain that, like the Pro-Q® Diamond gel stain, is quantitative over three orders of magnitude. Determining the ratio of the Pro-Q® Diamond dye to SYPRO® Ruby dye signal intensities for each band or spot thus provides a measure of the phosphorylation level normalized to the total amount of protein. Using both stains in combination makes it possible to distinguish a low amount of a highly phosphorylated protein from a higher amount of a less phosphorylated protein. To make this staining more convenient and economical, we offer the Multiplexed Proteomics’ Kit #2 with 200 mL of the Pro-Q® Diamond phosphoprotein gel stain and 200 mL of the SYPRO® Ruby protein gel stain (M33306), the Multiplexed Proteomics’ Kit #1 with 1 L of each stain (M33305) and the Multiplexed Proteomics’ Phosphoprotein Gel Stain Kits (MPP33305, MPM33306), which include the Pro-Q® Diamond phosphoprotein gel stain, SYPRO® Ruby protein gel stain and PeppermintStick™ phosphoprotein molecular weight standards; Table 9.4 summarizes all of our Pro-Q® Diamond gel stain reagents and kits.

**Table 9.4 Pro-Q® Diamond gel stain reagents and kits.**

<table>
<thead>
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<th>Product</th>
<th>Cat. No.</th>
<th>Pro-Q® Diamond Phosphoprotein Gel Stain</th>
<th>SYPRO® Ruby Protein Gel Stain</th>
<th>Phosphoprotein MW Standard</th>
<th>Number of Gels Stained</th>
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<td>Pro-Q® Diamond phosphoprotein gel stain</td>
<td>P33300</td>
<td>1 L</td>
<td></td>
<td></td>
<td>~20 minigels or two large-format gels (e.g., 2D gels)</td>
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<td>Pro-Q® Diamond phosphoprotein gel stain</td>
<td>P33301</td>
<td>200 mL</td>
<td></td>
<td></td>
<td>~4 minigels</td>
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<tr>
<td>Pro-Q® Diamond phosphoprotein gel stain</td>
<td>P33302</td>
<td>5 L</td>
<td></td>
<td></td>
<td>~100 minigels or 10 large-format gels</td>
</tr>
<tr>
<td>Multiplexed Proteomics® Phosphoprotein Gel Stain Kit #1</td>
<td>M33305</td>
<td>1 L</td>
<td>1 L</td>
<td></td>
<td>~20 minigels or two large-format gels</td>
</tr>
<tr>
<td>Multiplexed Proteomics® Phosphoprotein Gel Stain Kit #2</td>
<td>M33306</td>
<td>200 mL</td>
<td>200 mL</td>
<td></td>
<td>~4 minigels</td>
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<tr>
<td>Pro-Q® Diamond Phosphoprotein Gel Staining Kit</td>
<td>MPP33300</td>
<td>1 L</td>
<td>40 µL</td>
<td></td>
<td>~20 minigels or two large-format gels</td>
</tr>
<tr>
<td>Pro-Q® Diamond Phosphoprotein Gel Staining Kit</td>
<td>MPP33301</td>
<td>200 mL</td>
<td>40 µL</td>
<td></td>
<td>~4 minigels</td>
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<tr>
<td>Pro-Q® Diamond Phosphoprotein Gel Staining Kit</td>
<td>MPP33302</td>
<td>5 L</td>
<td>40 µL</td>
<td></td>
<td>~100 minigels or 10 large-format gels</td>
</tr>
<tr>
<td>Multiplexed Proteomics® Phosphoprotein Gel Stain Kit</td>
<td>MPM33305</td>
<td>1 L</td>
<td>1 L</td>
<td>40 µL</td>
<td>~20 minigels or two large-format gels</td>
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<tr>
<td>Multiplexed Proteomics® Phosphoprotein Gel Stain Kit</td>
<td>MPM33306</td>
<td>200 mL</td>
<td>200 mL</td>
<td>40 µL</td>
<td>~4 minigels</td>
</tr>
</tbody>
</table>

**Pro-Q® Diamond Phosphoprotein Blot Stain Kit**

The Pro-Q® Diamond Phosphoprotein Blot Stain Kit (P33356) provides a simple and quick method for directly detecting phosphoproteins on poly(vinylidene difluoride) (PVDF) or nitrocellulose membranes without the use of radioactivity or antibodies. As with the gel stain, the Pro-Q® Diamond phosphoprotein blot stain detects phosphoserine-, phosphothreonine- and phosphotyrosine-containing proteins, independent of the sequence context of the phosphorylated amino acid residue. Thus, the native phosphorylation levels of proteins from a variety of sources, including tissue specimens and body fluids, can be analyzed. Protein samples are separated by 1D or 2D gel electrophoresis, electroblotted to the membrane, stained and destained using a protocol similar to that typically performed with amido black or Ponceau S staining of total-protein profiles on membranes. After staining, gels are simply imaged using any of a variety of laser scanners, xenon-arc lamp–based scanners or CCD-based imaging devices employing UV transilluminators; the excitation/emission maxima of the Pro-Q® Diamond phosphoprotein blot stain are ~555/580 nm (Figure 9.4.9). The limits of detection for the stain on PVDF membrane blots are typically 8–16 ng of phosphoprotein, with a linear dynamic range of approximately 15-fold. The sensitivity of the Pro-Q® Diamond phosphoprotein blot stain is decreased when using nitrocellulose blots. Each Pro-Q® Diamond Phosphoprotein Blot Stain Kit provides sufficient reagents for staining ~20 minigel electrobLOTS, including:

- Pro-Q® Diamond phosphoprotein blot stain reagent
- Pro-Q® Diamond blot stain buffer
- Detailed protocols for staining and photographing the blot
The Pro-Q® Diamond phosphoprotein blot stain binds noncovalently to phosphoproteins and is thus fully compatible with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and Edman sequencing. Furthermore, the Pro-Q® Diamond phosphoprotein blot stain is compatible with the standard colorimetric, fluorometric and chemiluminescent detection techniques employed in immunoblotting. This phosphoprotein blot stain may be used in conjunction with the SYPRO® Ruby protein blot stain, a total-protein stain that is quantitative over two orders of magnitude on blots. Using the SYPRO® and Pro-Q® Diamond blot stains in combination makes it possible to distinguish a low amount of a highly phosphorylated protein from a higher amount of a less phosphorylated protein.

**PeppermintStick™ Phosphoprotein Molecular Weight Standards**

PeppermintStick™ phosphoprotein molecular weight standards are a mixture of phosphorylated and nonphosphorylated proteins with molecular weights from 14,400 to 116,250 daltons. Separation by polyacrylamide gel electrophoresis resolves this mixture into two phosphorylated and four nonphosphorylated protein bands (Figure 9.4.10). These standards serve both as molecular weight markers and as positive and negative controls for our Pro-Q® Diamond phosphoprotein gel stain and other methods that detect phosphorylated proteins. We offer two different unit sizes of the PeppermintStick™ phosphoprotein molecular weight standards: a 40 µL unit size sufficient for 20–40 gel lanes (P27167) and a 400 µL unit size sufficient for 200–400 gel lanes (P33350).

**Phosphopeptide Standard Mixture**

Formulated especially for MALDI-MS, the phosphopeptide standard mixture (P33357) contains equimolar amounts of three unphosphorylated and four phosphorylated peptides, ranging in mass between 1047 and 2192 and representing phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) monophosphopeptides, as well as a peptide containing both pT and pY. This mixture is ideal for use as an internal or external control for LC/MS, MALDI analysis or β-elimination reactions.

**Pro-Q® Diamond Phosphoprotein Enrichment Kits**

The Pro-Q® Diamond Phosphoprotein Enrichment Kit (P33358) enables efficient, nonradioactive isolation of phosphoproteins from complex cellular extracts. This kit provides resin, reagents and columns designed to isolate phosphoproteins from 0.5–1.0 mg of total cellular protein per column. The column bed volume can be easily scaled up or down depending on the amount of available starting material. The phosphoprotein-binding properties of the resin allow efficient capture of both native and denatured proteins. Therefore, cell or tissue samples can be denatured in lysis buffers and stored in the freezer prior to the phosphoprotein enrichment procedure. Each Pro-Q® Diamond Phosphoprotein Enrichment Kit contains:

- Phosphoprotein Enrichment Module
- Resin (50% v/v slurry)
- Disposable 2 mL columns, 10 columns
- Lysis buffer
- Wash buffer
- Elution buffer
- Vivasin® filtration concentrators with 10 kDa cut-off polyethersulfone membrane, 10 concentrators
- Protease Inhibitor and Endonuclease Module
- Protease inhibitor
- Endonuclease

Protocols for both undenatured and denatured lysates are provided, and these procedures can be completed in approximately three hours. For added convenience, the Pro-Q® Diamond Phosphoprotein Enrichment and Detection Kit (P33359) provides all the reagents in the Pro-Q® Diamond Phosphoprotein Enrichment Kit, as well as Pro-Q® Diamond phosphoprotein gel stain and PeppermintStick™ phosphoprotein molecular weight markers for detecting phosphoproteins on SDS-polyacrylamide gels.

**Pro-Q® Diamond Phosphoprotein/Phosphopeptide Microarray Stain Kit**

The Pro-Q® Diamond Phosphoprotein/Phosphopeptide Microarray Stain Kit (P33706) provides a method for selective staining of phosphoproteins or phosphopeptides on microarrays, without the use of antibodies or radioactivity. This kit permits direct detection of phosphate groups attached to tyrosine, serine or threonine residues in a microarray environment and has been optimized for microarrays with acrylamide gel surfaces. Each Pro-Q® Diamond Phosphoprotein/Phosphopeptide Microarray Stain Kit provides:

- Pro-Q® Diamond phosphoprotein/phosphopeptide microarray stain
- Pro-Q® Diamond microarray destain solution
- Microarray staining gasket with seal tabs, 10 chambers
- Slide holder tube, 20 tubes
- Detailed protocols

The Pro-Q® Diamond Phosphoprotein/Phosphopeptide Microarray Stain Kit is useful for identifying kinase targets in signal transduction pathways and for phosphoproteomics studies.
**Pro-Q® Diamond LC Phosphopeptide Detection Kit**

The Pro-Q® Diamond LC Phosphopeptide Detection Kit (P33203) is designed to provide sensitive and selective fluorescence-based detection of phosphorylated peptides during liquid chromatography separations. The Pro-Q® Diamond LC phosphopeptide detection reagent interacts selectively with phosphoserine-, phosphothreonine- and phosphotyrosine-containing peptides to form highly fluorescent dye-phosphopeptide complexes that elute from an HPLC column with altered retention times, allowing identification and purification of phosphopeptides prior to analysis by mass spectrometry. This kit is ideal for isolating phosphopeptides from chromatographic fractions or from complex peptide mixtures such as the tryptic digest of a phosphoprotein. The Pro-Q® Diamond LC Phosphopeptide Detection Kit provides:

- Pro-Q® Diamond LC phosphopeptide detection reagent
- Concentrated activation buffer
- Positive control phosphopeptide RII
- Kemptide, a negative control peptide
- Detailed protocols

Sufficient reagents are provided for 20 HPLC separations; a single separation will selectively detect 20 picomoles or less of a monophosphorylated peptide using a standard microbore C18 HPLC column.

**Other Reagents for Phosphoproteomics**

The Antibody Beacon™ Tyrosine Kinase Assay Kit (A35725), described in detail in Section 10.3, provides a simple yet robust solution assay for real-time monitoring of tyrosine kinase activity and the effectiveness of potential inhibitors and modulators. The key to this tyrosine kinase assay is a small-molecule tracer ligand labeled with our bright green-fluorescent Oregon Green® 488 dye. When an anti-phosphotyrosine antibody binds this tracer ligand to form the Antibody Beacon™ detection complex, the fluorescence of the Oregon Green® 488 dye is efficiently quenched. In the presence of a phosphotyrosine-containing peptide, however, this Antibody Beacon™ detection complex is rapidly disrupted, releasing the tracer ligand and relieving its antibody-induced quenching. Upon its displacement by a phosphotyrosine residue, the Oregon Green® 488 dye–labeled tracer ligand exhibits an approximately 4-fold enhancement in its fluorescence, enabling the detection of as little as 1 ng of a glycoprotein per band in gels (4 ng/band with the Pro-Q® Emerald 488 stain), making these stains about 50 times more sensitive than the standard periodic acid–Schiff base method using acidic fuchsin dye. Blot staining is not quite as sensitive (2–18 ng of a glycoprotein per band can be detected) and is more time consuming, but provides an opportunity to combine glycoprotein staining with immunostaining or other blot-based detection techniques. The Pro-Q® Emerald 300 stain is best visualized using 300 nm UV illumination, whereas the Pro-Q® Emerald 488 stain is best visualized using visible light with wavelengths near its 510 nm excitation maximum. The Pro-Q® Emerald dye is also used as the detection reagent in our Pro-Q® Emerald 300 Lipopolysaccharide Gel Stain Kit (P20495), which is described in Section 13.3.

**Pro-Q® Glycoprotein Stain Kits for Gels and Blots**

Glycoproteins play important roles as cell-surface markers, as well as in cell adhesion, immune recognition and inflammation reactions. To facilitate research on glycoproteins, we offer the Pro-Q® Glycoprotein Stain Kits for Gels and for Blots, which provide extraordinary sensitivity, linearity and ease of use for selective detection of glycoproteins.

**Pro-Q® Emerald Glycoprotein Stain Kits for Gels and for Blots**

Pro-Q® Emerald 300 and Pro-Q® Emerald 488 Glycoprotein Stain Kits (Table 9.5) provide advanced reagents for detecting glycoproteins in gels and on blots. The Pro-Q® Emerald glycoprotein stains react with periodate-oxidized carbohydrate groups, creating a bright green-fluorescent signal on glycoproteins (Figure 9.4.11). The staining procedure requires only three steps: fixation, oxidation and staining—no reduction step is required. Depending on the nature and degree of glycosylation, the Pro-Q® Emerald 300 stain allows the detection of as little as 1 ng of a glycoprotein per band in gels (4 ng/band with the Pro-Q® Emerald 488 stain), making these stains about 50 times more sensitive than the standard periodic acid–Schiff base method using acidic fuchsin dye. Blot staining is not quite as sensitive (2–18 ng of a glycoprotein per band can be detected) and is more time consuming, but provides an opportunity to combine glycoprotein staining with immunostaining or other blot-based detection techniques. The Pro-Q® Emerald 300 stain is best visualized using 300 nm UV illumination, whereas the Pro-Q® Emerald 488 stain is best visualized using visible light with wavelengths near its 510 nm excitation maximum. The Pro-Q® Emerald dye is also used as the detection reagent in our Pro-Q® Emerald 300 Lipopolysaccharide Gel Stain Kit (P20495), which is described in Section 13.3.

**Table 9.5 Pro-Q® Emerald glycoprotein stain kits for gels and for blots.**

<table>
<thead>
<tr>
<th>Product</th>
<th>Glycoprotein Stain</th>
<th>Kit Type</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-Q® Emerald 300 Glycoprotein Stain Kits</td>
<td>Pro-Q® Emerald 300 stain, Ex/Em = 280/530 nm</td>
<td>Gel Stain Kit (includes SYPRO® Ruby protein gel stain *)</td>
<td>P21855</td>
</tr>
<tr>
<td>Pro-Q® Emerald 488 Glycoprotein Stain Kit</td>
<td>Pro-Q® Emerald 488 stain, Ex/Em = 510/520 nm</td>
<td>Gel and Blot Stain Kit (does not include a total-protein stain)</td>
<td>P21875</td>
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</tbody>
</table>

* See Section 9.3 for a description of the SYPRO® Ruby protein gel stain.
The Pro-Q® Emerald glycoprotein stains can be combined with general protein stains for dichromatic detection of glycoproteins and total proteins in gels and on blots, making it much easier to identify the location of the glycoproteins in the total-protein profile (Figure 9.4.12, Figure 9.4.13, Figure 9.4.14, Figure 9.4.15). The SYPRO® Ruby protein gel and blot stains (described in Section 9.3) provide the same sensitivity as silver staining (gels) or colloidal gold staining (blots) but, unlike these chromogenic techniques, do not require formaldehyde or glutaraldehyde, which can produce false-positive responses when glycoproteins are stained. These total-protein stains make it possible to visualize the entire protein complement of a sample and to thus identify contaminating proteins, to compare stained proteins to molecular weight standards and to provide a control for protease contamination in glycosidase mobility-shift experiments. The SYPRO® Ruby protein blot stain is additionally useful for assessing the efficiency of protein transfer to a blot (Figure 9.4.14), which is especially important when working with glycoproteins, because they often transfer poorly to blotting membranes. Proteins labeled with the SYPRO® Ruby total-protein stains exhibit red-orange fluorescence when excited with either a 300 nm UV light source or a laser scanner with a 473, 488 or 532 nm laser light source.

The Pro-Q® Emerald Glycoprotein Stain Kits also include our CandyCane™ molecular weight standards, a mixture of glycosylated and nonglycosylated proteins that, when separated by electrophoresis, provide alternating positive and negative controls (Figure 9.4.16). The CandyCane™ molecular weight standards are also available separately (C21852). In addition, we offer a Pro-Q®
Emerald 300 Glycoprotein Gel Stain Kit (P21855) that includes our SYPRO® Ruby protein gel stain for detecting total proteins. The Pro-Q® Emerald Glycoprotein Stain Kits for gels and blots (P21855, P21857, P21875) contain sufficient materials to stain approximately ten 8 cm × 10 cm gels or blots, including:

- Pro-Q® Emerald 300 glycoprotein stain (in Kits P21855 and P21857)
- Pro-Q® Emerald 488 glycoprotein stain (in Kit P21875)
- Pro-Q® Emerald 300 staining buffer (in Kits P21855 and P21857 or Pro-Q® Emerald 488 staining buffer (in Kit P21875)
- Oxidizing reagent (periodic acid)
- SYPRO® Ruby protein gel stain (in Kit P21855 only)
- CandyCane™ glycoprotein molecular weight standards
- Detailed protocols

### Multiplexed Proteomics® Kit for Glycoprotein and Total-Protein Gel Staining

When used together, the Pro-Q® Emerald 300 glycoprotein gel stain and the SYPRO® Ruby protein gel stain (S12000, S12001, S21900; Section 9.3) make a powerful combination for proteome analysis (Figure 9.4.12). Determining the ratio of the Pro-Q® Emerald dye to SYPRO® Ruby dye signal intensities for each band or spot provides a measure of the glycosylation level normalized to the total amount of protein. Using both stains in combination makes it possible to distinguish a lightly glycosylated, high-abundance protein from a heavily glycosylated, low-abundance protein. To make this staining more convenient and economical, we offer the Multiplexed Proteomics® Glycoprotein Gel Stain Kit with 1 L of our Pro-Q® Emerald 300 glycoprotein gel stain and 1 L of our SYPRO® Ruby protein gel stain (M33307).

### CandyCane™ Glycoprotein Molecular Weight Standards

CandyCane™ glycoprotein molecular weight standards (C21852) contain a mixture of glycosylated and nonglycosylated proteins with molecular weights from 14,000 to 180,000 daltons. When separated by polyacrylamide gel electrophoresis, the standards appear as alternating bands corresponding to glycosylated and nonglycosylated proteins (Figure 9.4.16). Thus, these standards serve both as molecular weight markers and as positive and negative controls for methods that detect glycosylated proteins, such as those provided in our Pro-Q® Emerald Glycoprotein Stain Kits.

### Other Specialized Techniques for Detecting Specific Proteins on Gels and Blots

#### Detecting Calcium-Binding Proteins

The luminescent lanthanide terbium, which is available as its chloride salt (Tb³⁺ from TbCl₃, T1247), selectively stains calcium-binding proteins in SDS-polyacrylamide gels. With some modifications to the staining protocol, these lanthanides can also be used to detect all protein bands. Terbium chloride has also been used as a rapid negative stain for proteins in SDS-polyacrylamide gels, in which the background is green fluorescent and the proteins are unstained.

#### Detecting Penicillin-Binding Proteins

BOCILLIN™ FL penicillin and BOCILLIN™ 650/665 penicillin (B13233, B13234) are green- and infrared-fluorescent penicillin analogs, respectively, that bind selectively and with high affinity to penicillin-binding proteins in SDS-polyacrylamide gels. With some modifications to the staining protocol, these analogs are green- and infrared-fluorescent, respectively, that bind selectively and with high affinity to penicillin-binding proteins in SDS-polyacrylamide gels.

### Figure 9.4.14 Staining glycoproteins and the total protein profile on blots using the Pro-Q® Emerald 300 Glycoprotein Gel and Blot Stain Kit (P21857). A 2-fold dilution series of the CandyCane™ glycoprotein molecular weight standards (C21852) was run an SDS-polyacrylamide gel and blotted onto a PVDF membrane. The blot was first stained with the SYPRO® Ruby protein gel stain (S11791) to detect the total protein profile (left). After documentation of the signal, the blot was stained with the Pro-Q® Emerald 300 glycoprotein stain (right) provided in the Pro-Q® Emerald 300 Glycoprotein Gel and Blot Stain Kit.

### Figure 9.4.15 2D gel stained with the SYPRO® Ruby protein gel stain and the Pro-Q® Emerald 300 reagent. Combined Cohn fractions II and III from cow plasma, containing primarily B- and γ-globulins, were run on a 2D gel and stained first with the Pro-Q® Emerald 300 reagent (P21855, P21857; left) and then with the SYPRO® Ruby protein gel stain (S12000, S12001, S21900, and in P21855; right).

### Figure 9.4.16 Glycosylated and nonglycosylated proteins in the CandyCane™ glycoprotein molecular weight standards (C21852). The standards were electrophoresed through two identical 13% polyacrylamide gels. Both lanes contain ~0.5 µg of protein in each band. The left lane was stained with our SYPRO® Ruby protein gel stain (S12000, S12001, S21900) to detect all eight marker proteins. The right lane was stained using the reagents in the Pro-Q® Emerald 300 Glycoprotein Gel Stain Kit (P21855).
penicillin-binding proteins present on the cytoplasmic membranes of eubacteria.\(^{33,35}\) When electrophoresed under nonreducing conditions, the dye-labeled penicillin-binding proteins are easily visible in the gel with sensitivity in the low nanogram range\(^{34}\) (Figure 9.4.17). BOCILLIN™ FL penicillin, synthesized from penicillin V and the BODIPY® FL dye (spectrally similar to fluorescein), has been used to determine the penicillin-binding protein profiles of *Escherichia coli*, *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*, and these binding profiles are found to be similar to those reported by researchers using radioactively labeled penicillin.\(^{32}\) Fluorescently labeled penicillin has also been used for direct labeling and rapid detection of whole *E. coli* and *Bacillus licheniformis*\(^{35}\) and of *Enterobacter pneumoniae*.\(^{36}\)

The β-lactam sensor-transducer (BlaR), an integral membrane protein from *Staphylococcus aureus*, covalently and stoichiometrically reacts with β-lactam antibiotics, including BOCILLIN™ FL penicillin, by acylation of its active-site serine residue.\(^{37}\)

**Detecting TC-Tagged Fusion Proteins with TC-FlAsH™ and TC-ReAsH™ Reagents**

TC-FlAsH™ and TC-ReAsH™ detection technology, based on the tetracysteine tag first described by Griffin, Adams and Tsien in 1998,\(^{38,39}\) takes advantage of the high-affinity interaction of a biarsenical ligand (FlAsH-EDT\(_2\) or ReAsH-EDT\(_2\)) with the thiols in a tetracysteine (TC) expression tag fused to the protein of interest. The FlAsH-EDT\(_2\) ligand is essentially fluorescent that has been modified to contain two arsonium atoms at a set distance from each other, whereas the ReAsH-EDT\(_2\) ligand is a similarly modified resorufin. Virtually nonfluorescent in the ethanedithiol (EDT)-bound state, these reagents become highly fluorescent when bound to the thiol-containing tetracysteine tag Cys-Cys-Xxx-Yyy-Cys-Cys, where Xxx-Yyy is typically Pro-Gly\(^{40}\) (Figure 9.4.18). Modified tags with additional flanking sequences produce higher affinity binding of the biarsenical ligand, resulting in improved signal-to-background characteristics.\(^{41,42}\)

Transduction of the host cell line with an expression construct comprising the protein of interest fused to a tetracysteine tag is followed by addition FlAsH-EDT\(_2\) reagent (Figure 9.4.18) or ReAsH-EDT\(_2\) reagent, generating green or red fluorescence, respectively, upon binding the tetracysteine motif. For detection of tetracysteine-tagged proteins expressed in cells, we offer the TC-FlAsH™ II and TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits (T34561, T34562, T34563), which are described in Section 2.2.

As an alternative to in-cell detection, we offer the TC-FlAsH™ Expression Analysis Detection Kits (A10067, A10068) for detecting tetracysteine-tagged proteins in polyacrylamide gels (Figure 9.4.19). These kits provide:

- FlAsH loading buffer
- Orange total-protein stain (in A10067) or Red total-protein stain (in A10068)
- Dimethylsulfoxide (DMSO)
- BenchMark™ Protein Ladder
- Detailed protocols

Sufficient reagents are provided for ten 17-well minigels, based on a 12 µL reaction volume. When bound to TC-tagged protein, FlAsH dye exhibits excitation/emission maxima of 505/530 nm. The Orange and Red total-protein gel stains supplied in these detection kits exhibit emission maxima of 585/620 nm and 650/660 nm, respectively.

![Figure 9.4.19](image_url) Protein gel staining using TC-FlAsH™ Expression Analysis Detection Kit (A10068). A) Tetracysteine-tagged proteins are labeled with FlAsH-EDT\(_2\) reagent and fluoresce green. B) Total proteins are labeled with the Red total-protein stain provided in the kit and fluoresce red. C) An overlay of the two images reveals relative amounts of protein.
Detecting Oligohistidine Fusion Proteins

The oligohistidine domain is a Ni²⁺-binding peptide sequence comprising a string of four to six histidine residues. When the DNA sequence corresponding to the oligohistidine domain is fused in frame with a gene of interest, the resulting recombinant protein can be easily purified using a nickel-chelating resin.⁴¹,⁴⁴

Developed by QIAGEN, the Penta·His mouse IgG₁ monoclonal antibody (P21315) provides a sensitive method for specific detection of fusion proteins that have an oligohistidine domain comprising five or six consecutive histidine residues. The antibody does not recognize tetrahistidine domains or domains in which the histidine string is interrupted by another amino acid. The Penta·His antibody binds to the oligohistidine domain regardless of the surrounding amino acid context and even when the group is partially hidden, although subtle differences in the amino acid context may change the sensitivity limit for a particular fusion protein. The antibody is ideal for detecting oligohistidine fusion proteins on western blots (Figure 9.4.20). The Penta·His antibody is also useful for immunoprecipitation, ELISA assays and immunohistochemistry.

Anti-Dinitrophenyl Antibody 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 2,4-dinitrophenylhydrazine to yield protein-and ketones.⁴⁵ Traditionally, protein-derived aldehydes and ketones have been quantitated using a sensitive method for speci

Biotinylated glutathione ethyl ester (BioGEE, G36000; Figure 9.4.22) is a cell-permeant, biotinylated glutathione analog for detecting glutathiolation. Under conditions of oxidative stress, cells may transiently incorporate glutathione into proteins. Stressed cells incubated with BioGEE will also incorporate this biotinylated glutathione derivative into proteins, facilitating the identification of oxidation-sensitive proteins.⁴⁷,⁴⁸ Once these cells are fixed and permeabilized, glutathiolation levels can be detected with a fluorescent streptavidin conjugate (Table 7.9) using either flow cytometry or fluorescence microscopy. Proteins glutathiolated with BioGEE can be captured using streptavidin or CaptAvidin™ agarose (S951, C21386; Section 7.6) and analyzed by mass spectrometry or by western blotting methods.⁴⁹,⁵⁰

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

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), comprising six histidine residues. When the DNA sequence corresponding to the oligohistidine domain is fused with the overexpressed oligomycin sensitivity–conferring protein (OSCP) fused with a hexahistidine domain were run on an SDS-polyacrylamide gel and blotted onto a PVDF membrane. The blot was stained with the SYPRO® Ruby protein blot stain (S11791) to detect the entire protein profile (top). After imaging, the blot was incubated with Penta·His mouse IgG₁ monoclonal antibody (P21315), followed by immunodetection using an alkaline phosphatase conjugate of goat anti–mouse antibody in conjunction with DDAO phosphate (bottom).
a protein with natural binding specificity that can be exploited to facilitate its purification.51 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.32–35 (Figure 9.4.23). For purification of GST fusion proteins, we offer glutathione linked via the sulfur atom to crosslinked beaded agarose (10 mL of sedimented bead suspension, G2879). 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 recovered by washing with a high-salt buffer.

We also offer a highly purified rabbit polyclonal anti-GST antibody (A5800) that can be used to purify GST fusion proteins by immunoprecipitation.56 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 and for detecting GST distribution in cells. The intensely green-fluorescent Alexa Fluor® 488 conjugate of anti-glutathione S-transferase (A11131) is also available for direct detection of GST fusion proteins.

Following purification, the fusion protein can serve as an immunogen for antibody production54,56 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,61 the role of the Rho GTP-binding protein in 1bc oncogene function62 and the association of v-Src with cortactin in Rous sarcoma virus–transformed cells.63 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.64 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.52 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.65–68 Once the fusion protein is bound to the affinity matrix, the site-specific enzyme can be added to release the protein.

Streptavidin Acrylamide and Reactive Acrylamide Derivatives

Streptavidin acrylamide (S21379), which is prepared from the succinimidyl ester of 6-((acryloyl)amino)hexanoic acid (acryloyl-X, SE, A20770), may be useful for the preparation of biosensors.69 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.

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. We prepare both streptavidin and CaptAvidin™ biotin-binding protein conjugated to 4% beaded crosslinked agarose (S951, C21386; Section 7.6)—matrices that can be used to isolate biotinylated peptides, proteins, hybridization probes, haptons and other molecules.
### PRODUCT LIST 9.4 DETECTING PROTEIN MODIFICATIONS

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<th>Cat. No.</th>
<th>Product Description</th>
<th>Quantity</th>
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</thead>
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<td>6-(acryloylamo)hexanoic acid, succinimidyl ester (acryloyl-X, SE)</td>
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<td>A5800</td>
<td>anti-glutathione S-transferase, rabbit IgG fraction <em>3 mg/mL</em></td>
<td>0.5 mL</td>
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<td>anti-glutathione S-transferase, rabbit IgG fraction, Alexa Fluor® 488 conjugate <em>2 mg/mL</em></td>
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<td>BOCILLIN® 650/665 penicillin, sodium salt</td>
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<td>B13233</td>
<td>BOCILLIN™ FL penicillin, sodium salt</td>
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<tr>
<td>C21852</td>
<td>CandyCane™ glycoprotein molecular weight standards <em>200 gel lanes</em></td>
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<td>C10102</td>
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<td>Click-tT AHA Alexa Fluor® 488 Protein Synthesis HCS Assay &quot;2-plate size&quot;</td>
<td>1 kit</td>
</tr>
<tr>
<td>C33372</td>
<td>Click-tT Biotin Protein Analysis Detection Kit &quot;10 reactions&quot;</td>
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</tr>
<tr>
<td>C10269</td>
<td>Click-tT Cell Reaction Buffer Kit</td>
<td>1 kit</td>
</tr>
<tr>
<td>C33371</td>
<td>Click-tT Dapsyl® Protein Analysis Detection Kit &quot;for UV excitation&quot; &quot;10 reactions&quot;</td>
<td>1 kit</td>
</tr>
<tr>
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<td>Click-tT farnesyl alcohol, azide &quot;mixed isomers&quot;</td>
<td>1 mg</td>
</tr>
<tr>
<td>C10264</td>
<td>Click-tT fucose alkyne (tetraacetylfluoresce alkyne)</td>
<td>5 mg</td>
</tr>
<tr>
<td>C33365</td>
<td>Click-tT GaINaZ metabolic glycoprotein labeling reagent (tetracyctealted N-azidoacetylgalactosamine) &quot;for O-linked glycoproteins&quot; <em>5.2 mg</em></td>
<td>1 each</td>
</tr>
<tr>
<td>C10249</td>
<td>Click-tT geranylgeranyl alcohol, azide &quot;mixed isomers&quot;</td>
<td>1 mg</td>
</tr>
<tr>
<td>C33368</td>
<td>Click-tT O-GlcNAc Enzymatic Labeling System &quot;for O-linked GlcNAc glycoproteins&quot; <em>10 labelings</em></td>
<td>1 kit</td>
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<td>C33373</td>
<td>Click-tT O-GlcNAc peptide and phosphopeptide LC/MS standards <em>5 nmol each</em></td>
<td>1 set</td>
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<td>C33374</td>
<td>Click-tT O-GlcNAc peptide LC/MS standard (H-Thr-Ala-Pro-Thr-D-GlcNAcSer-Thr-Ile-Ala-Pro-Gly-OH) &quot;Theoretical Mass (M+H) 1118.50&quot;</td>
<td>5 nmol</td>
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<tr>
<td>C33367</td>
<td>Click-tT GlcNAZ metabolic glycoprotein labeling reagent (tetracyctealted N-azidoacetylgluconsamine) &quot;for O-GlcNAc-modified proteins&quot; <em>5.2 mg</em></td>
<td>1 each</td>
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<td>C33366</td>
<td>Click-tT ManNaZ metabolic glycoprotein labeling reagent (tetracyctealted N-azidoacetyld-mannosamine) &quot;for sialic acid glycoproteins&quot; <em>5.2 mg</em></td>
<td>1 each</td>
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<td>C10268</td>
<td>Click-tT myristic acid, azide (12-azido-dodecanoic acid)</td>
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<tr>
<td>C10265</td>
<td>Click-tT palmityl acid, azide (15-azido-pentadecanoic acid)</td>
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</tr>
<tr>
<td>C33370</td>
<td>Click-tT Tetramethylrhodamine (TAMRA) Protein Analysis Detection Kit &quot;UV/532 nm excitation&quot; &quot;10 reactions&quot;</td>
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<tr>
<td>G2879</td>
<td>glutathione agarse, linked through sulfur &quot;sedimented bead suspension&quot;</td>
<td>10 mL</td>
</tr>
<tr>
<td>G36000</td>
<td>glutathione ethyl ester, biotin amide (BioGEE) &quot;glutathiolation detection reagent&quot; &quot;special packaging&quot;</td>
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<td>1 kit</td>
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<tr>
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<td>1 kit</td>
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<tr>
<td>MMP33306</td>
<td>Multiplexed Proteomics™ Phosphoprotein Gel Stain Kit &quot;includes MMP33301 and S12001&quot;</td>
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<td>M33305</td>
<td>Multiplexed Proteomics™ Phosphoprotein Gel Stain Kit #1 &quot;with 1 L of each of Pro-Q® Diamond (P33300) and SYPRO® Ruby (S12000) gel stains&quot;</td>
<td>1 set</td>
</tr>
<tr>
<td>M33306</td>
<td>Multiplexed Proteomics™ Phosphoprotein Gel Stain Kit #2 &quot;with 200 mL of each of Pro-Q® Diamond (P33300) and SYPRO® Ruby (S12001) gel stains&quot;</td>
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</tr>
<tr>
<td>P21315</td>
<td>Penta-His mouse IgG, monoclonal antibody (anti-pentahistidine) &quot;BSA free&quot;</td>
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</tr>
<tr>
<td>P21767</td>
<td>PeppermintStick™ phosphoprotein molecular weight standards</td>
<td>40 µL</td>
</tr>
<tr>
<td>P33350</td>
<td>PeppermintStick™ phosphoprotein molecular weight standards &quot;200 gel lanes&quot;</td>
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</tr>
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<td>P33357</td>
<td>phosphopeptide standard mixture &quot;400 pmol of each peptide&quot;</td>
<td>2600 pmol</td>
</tr>
<tr>
<td>P33320</td>
<td>Pro-Q® Diamond LC Phosphopeptide Detection Kit</td>
<td>1 kit</td>
</tr>
<tr>
<td>P33356</td>
<td>Pro-Q® Diamond Phosphoprotein Blot Stain Kit &quot;20 minigel blots&quot;</td>
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<tr>
<td>P33359</td>
<td>Pro-Q® Diamond Phosphoprotein Enrichment and Detection Kit</td>
<td>1 kit</td>
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<td>Pro-Q® Diamond Phosphoprotein Enrichment Kit</td>
<td>1 kit</td>
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<td>P33310</td>
<td>Pro-Q® Diamond phosphoprotein gel destaining solution</td>
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<td>P33311</td>
<td>Pro-Q® Diamond phosphoprotein gel destaining solution &quot;bulk packaging&quot;</td>
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</tr>
<tr>
<td>P33301</td>
<td>Pro-Q® Diamond phosphoprotein gel stain</td>
<td>200 mL</td>
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<tr>
<td>P33300</td>
<td>Pro-Q® Diamond phosphoprotein gel stain &quot;bulk packaging&quot;</td>
<td>5 L</td>
</tr>
<tr>
<td>MP33301</td>
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</tr>
<tr>
<td>MP33300</td>
<td>Pro-Q® Diamond Phosphoprotein Gel Staining Kit &quot;includes 1 L stain and 40 µL standard*&quot;</td>
<td>1 kit</td>
</tr>
<tr>
<td>MP33302</td>
<td>Pro-Q® Diamond Phosphoprotein Gel Staining Kit &quot;includes 5 L stain and 400 µL standard*&quot;</td>
<td>1 kit</td>
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<tr>
<td>P33306</td>
<td>Pro-Q® Diamond Phosphoprotein/Phosphopeptide Microarray Stain Kit</td>
<td>1 kit</td>
</tr>
<tr>
<td>P21857</td>
<td>Pro-Q® Emerald 300 Glycoprotein Gel and Blot Stain Kit &quot;10 minigel or minigel blots&quot;</td>
<td>1 kit</td>
</tr>
<tr>
<td>P21855</td>
<td>Pro-Q® Emerald 300 Glycoprotein Gel Stain Kit &quot;with SYPRO® Ruby protein gel stain&quot; <em>10 minigels</em></td>
<td>1 kit</td>
</tr>
<tr>
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<td>Pro-Q® Emerald 488 Glycoprotein Gel and Blot Stain Kit <em>10 minigels or minigel blots</em></td>
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<td>S21379</td>
<td>streptavidin acrylamide</td>
<td>1 mg</td>
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<td>A10067</td>
<td>TC-FIAx™ Expression Analysis Detection Kit - Orange &quot;fluorescent in-gel detection of TC-tagged and total protein&quot;</td>
<td>1 kit</td>
</tr>
<tr>
<td>A10068</td>
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<td>1 kit</td>
</tr>
<tr>
<td>T1247</td>
<td>terbium(lll) chloride, hexahydrate</td>
<td>1 g</td>
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9.5 Peptide Analysis, Sequencing and Synthesis

This section describes Molecular Probes® reagents used in the synthesis of fluorophore- or hapten-labeled peptides and fluorogenic protease substrates, as well as in peptide and protein sequencing. Some of our fluorescent probes and research chemicals have also been used for N-terminal amino acid analysis and peptide sequencing, as well as for protein fragment modification prior to Edman sequencing.

N-Terminal Amino Acid Analysis

Except when it is already blocked by formylation, acetylation, pyrogallamic acid formation or other chemistry, the N-terminal amino acid of proteins can be labeled with a variety of fluorescent and chromophoric reagents from Chapter 1. However, only those functional groups that survive complete protein hydrolysis, such as sulfonamides, are useful for N-terminal amino acid analysis. Dansyl chloride (D21) is the most commonly employed reagent for such analyses.

Nonacylated N-terminal serine and threonine residues of proteins can be periodate-oxidized to aldehydes \(^{(2-4)}\) (Figure 9.5.1) that can then be modiﬁed by a variety of hydrazine and hydroxylamine derivatives described in Section 3.3. Only peptides and proteins that contain these two terminal amino acids become fluorescent, although oxidation of the carbohydrate portion of glycoproteins to aldehydes may cause interference in this analysis.

N-Acetylated or N-formylated proteins have been detected by transfer of the acyl group to dansyl hydrazine (D100) and subsequent chromatographic separation of the ﬂuorescent product. The sensitivity of this method can likely be improved by the use of other fluorescent hydrazine and hydroxylamine derivatives described in Section 3.3.

Peptide Sequencing

The dominant chemistry for sequencing peptides employs the non-fluorescent reagent phenyl isothiocyanate, which forms phenylthiohydantoins (PTH) in the sequencing reaction. As analogs of phenyl isothiocyanate, fluorescein-5-isothiocyanate (FITC; F143, F1906, F1907; Section 1.5) and other ﬂuorescent isothiocyanates yield peptide conjugates that are also susceptible to Edman degradation via their thiocyanate group.

Terminal serine and -formylated proteins have been detected by terminal amino acid analysis and peptide sequencing, as well as for protein fragment modification prior to Edman sequencing.

Peptide Synthesis

Peptides specifically labeled with ﬂuorescent dyes, haptons, or radioactive groups or radioisotopes are important both as probes for receptors and as substrates for enzymes (Section 10.4). Labeled peptides can be prepared by modifying isolated peptides or by incorporating the label during solid-phase synthesis. We offer several ﬂuorescent neuropeptides, most of which are described in Section 16.2.

Labeling Peptides in Solution

Appropriately substituted synthetic peptides can be labeled in solution by almost any of the reactive probes described in Chapters 1–5 (Labeling Small Peptides with Amine-Reactive Dyes in Organic Solvents—Note 9.2). Many peptides contain multiple residues that can be modiﬁed, potentially leading to complex mixtures of products, some of which may be biologically inactive. Modiﬁcation of a peptide’s thiol group by one of the thiol-reactive reagents described in Chapter 2 is usually easy, selective and very efﬁcient. If the peptide is synthetic, or can be modiﬁed by site-directed mutagenesis, incorporation of a cysteine residue at the desired site of labeling is recommended. The N-terminal \(\alpha\)-amine of peptides, which has a lower pK\(_a\) than the \(\epsilon\)-amino group of lysine residues, can sometimes be labeled in the presence of other amines if the pH is kept near neutral. Conversion of tyrosine residues to \(\epsilon\)-aminotyrosines (Section 3.2) can be used to provide selective sites for peptide modiﬁcation, unless the tyrosine residues are essential for the biological activity of the peptide.

Solid-Phase Synthesis of Labeled Peptides

If speciﬁc labeling of peptides in solution is problematic, it may be more convenient to conjugate the ﬂuorophore to the N-terminus of a resin-bound peptide before removal of other protecting groups and release of the labeled peptide from the resin. About ﬁve equivalents of an amine-reactive ﬂuorophore are usually used per amine of the immobilized peptide. The ﬂuororescein, Alexa Fluor®, Oregon Green®, Rhodamine Green®, tetramethylrhodamine, Rhodamine Red®, Texas Red®, coumarin and NBD ﬂuorophores, as well as the QSY®, dabcyl and dabsyl chromophores and the biotin hapten, are all expected to be reasonably stable to hydrogen fluoride (HF) as well as to most other acids. These ﬂuorophores, chromophores and biotin are also expected to be stable to reagents used for deprotection of peptides synthesized using Fmoc chemistry. In contrast, the BODIPY® fluorophore may be unstable to the conditions used to remove some protecting groups.

We prepare unique reagents for use in the automated synthesis of peptides that are specifically labeled with fluorophores, chromophores and haptons. These precursors permit the incorporation of a speciﬁc label at a speciﬁc site in the peptide’s sequence. The \(\alpha\)-Fmoc derivative of \(\epsilon\)-dabcyl-L-lysine (D6216) can be used to incorporate the dabcyl chromophore at selected sites in the peptide sequence. The dabcyl chromophore, which has broad visible absorption (Figure 9.5.2), has been extensively used as a quenching group in the automated synthesis of HIV protease and renin (H2930, R2931; Section 10.4) and other fluorogenic peptides.
peptidase substrates. The dabcyl group can also be incorporated at the N-terminus by using dabcyl succinimidyl ester (D2245). The aminonaphthalene derivative EDANS (A91) has been the most common fluorophore for pairing with the dabcyl quencher in fluorescence resonance energy transfer (FRET) experiments because its fluorescence emission spectrum overlaps the absorption spectrum of dabcyl (Figure 9.5.2) (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2). This fluorophore is conveniently introduced during automated synthesis of peptides by using y-EDANS-a-FMOC-L-glutamic acid (F11831). The tetramethylrhodamine fluorophore can be incorporated during automated FMOc synthesis of peptides using our single-isomer ε-(FMOC)-ε-TMR-L-lysine building block (F11830).

QSY® dyes (Section 1.6, Section 1.8) have broad visible to near-infrared absorption (Figure 9.5.3). These dyes, which are essentially nonfluorescent, are particularly useful as energy acceptors from blue-, green-, orange- or red-fluorescent donor dyes (Note 1.2). The QSY® 7, QSY® 9, QSY® 21 and QSY® 35 chromophores can be conjugated to amines via their succinimidyl esters (Q10193, Q20131, Q20132, Q20133). The QSY® 7 and QSY® 35 chromophores can also be conjugated to peptide thiols or thiol-modified oligonucleotides via its maleimide (Q10257) or iodoacetamide (Q20348); additionally, peptide amides can be prepared from QSY® 7 amine (Q10464). We have also prepared α-(FMOC)-ε-QSY® 7-L-lysine and α-FMOC-β-QSY® 35-L-alanine (Q21930, Q21931), which can be used in the automated synthesis of QSY® 7 quencher– or QSY® 35 quencher–containing peptides.

REFERENCES

NOTE 9.2
Labeling Small Peptides with Amine-Reactive Dyes in Organic Solvents
Most of the product literature associated with our amine-reactive dyes provides protocols for labeling proteins, typically IgG antibodies in aqueous buffers. The following protocol is a starting point for labeling peptides in organic solvents. Please note that the reaction conditions, including concentrations of the reactants and the reaction times, may require optimization. Furthermore, many peptides are not soluble in a 100% organic solution. It is very important to test the solubility of the peptide in dimethylsulfoxide (DMSO) or dimethylformamide (DMF) before attempting this procedure.
1. Dissolve the peptide to be labeled in DMSO or DMF at 0.1–1 mM.
2. Add 100 mM triethylamine to the reaction solution. This will ensure that the amines to be derivatized are deprotonated.
3. Add the amine-reactive dye to the reaction solution. The reactive dye should be in a 1:1 to 3:1 molar ratio to the peptide.
4. React at room temperature or at 4°C for at least 4 hours with continuous stirring, protected from light. The reaction can proceed overnight. Thin-layer chromatography may be useful for monitoring the reaction’s progress.
5. Purify the conjugate by an appropriate method, such as HPLC-based separation.

Figure 9.5.2 Spectral overlap between EDANS fluorescence and dabcyl absorption, which is required for efficient quenching of EDANS fluorescence by resonance energy transfer to the nonfluorescent dabcyl chromophore. Spectra are normalized to the same intensities.

Figure 9.5.3 Normalized absorption spectra of the QSY® 35 (blue), QSY® 7 (red) and QSY® 21 (orange) dyes. The QSY® 7 and QSY® 9 dyes have essentially identical spectra.
### DATA TABLE 9.5 PEPTIDE ANALYSIS, SEQUENCING AND SYNTHESIS

<table>
<thead>
<tr>
<th>Cat. No.</th>
<th>MW</th>
<th>Storage</th>
<th>Soluble</th>
<th>Abs</th>
<th>EC</th>
<th>Em</th>
<th>Solvent</th>
<th>Notes</th>
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<tr>
<td>A91</td>
<td>288.30</td>
<td>L</td>
<td>pH &gt; 10, DMF</td>
<td>335</td>
<td>5900</td>
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<td>475</td>
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<td>none</td>
<td>MeOH</td>
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</table>

For definitions of the contents of this data table, see “Using The Molecular Probes® Handbook” in the introductory pages.

### Notes
1. D21 butylamine derivative has Abs = 337 nm (EC = 5300 cm⁻¹M⁻¹), Em = 492 nm in CHCl₃. Em and QY are highly solvent dependent: Em = 496 nm (QY = 0.45) in dioxane, 538 nm (QY = 0.28) in MeOH and 557 nm (QY = 0.03) in H₂O. (Biochemistry (1967) 6:3408) EC typically decreases upon conjugation to proteins (EC = 3400 cm⁻¹M⁻¹ at 340 nm). (Biochemistry (1986) 25:513) Fluorescence lifetimes (τ) of protein conjugates are typically 12–20 nanoseconds. (Arch Biochem Biophys (1969) 133:263, Arch Biochem Biophys (1968) 128:163)

2. Do NOT dissolve in DMSO.

3. D2245 is non-fluorescent both before and after reaction with amines. Reaction product with butylamine has Abs = 428 nm (EC = 32,000 cm⁻¹M⁻¹) in MeOH.

4. This sulfonated succinimidyl ester derivative is water soluble and may be dissolved in buffer at ~pH 8 for reaction with amines. Long-term storage in water is NOT recommended due to hydrolysis.

5. Iodoacetamides in solution undergo rapid photodecomposition to unreactive products. Minimize exposure to light prior to reaction.

### PRODUCT LIST 9.5 PEPTIDE ANALYSIS, SEQUENCING AND SYNTHESIS

<table>
<thead>
<tr>
<th>Cat. No.</th>
<th>Product</th>
<th>Quantity</th>
</tr>
</thead>
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<td>5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid, sodium salt (EDANS)</td>
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<tr>
<td>D21</td>
<td>5-dimethylaminonaphthalene-1-sulfonic chloride (dansyl chloride)</td>
<td>1 g</td>
</tr>
<tr>
<td>D100</td>
<td>5-dimethylaminonaphthalene-1-sulfonic hydrazine (dansyl hydrazine)</td>
<td>100 mg</td>
</tr>
<tr>
<td>D2245</td>
<td>4-(((dimethylamino)phenyl)azo)benzoic acid, succinimidyl ester (dabcyl, SE)</td>
<td>100 mg</td>
</tr>
<tr>
<td>D6216</td>
<td>4-(((4-dimethylamino)phenyl)azo)benzoyl-o-9-fluorenylmethoxycarbonyl-L-lysine (o-dabcyl-OMOC-L-lysine)</td>
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</tr>
<tr>
<td>F11830</td>
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<td>100 mg</td>
</tr>
<tr>
<td>F11831</td>
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<td>Q10464</td>
<td>QSY° 7 amine, hydrochloride</td>
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</tr>
<tr>
<td>Q10193</td>
<td>QSY° 7 carboxylic acid, succinimidyl ester</td>
<td>5 mg</td>
</tr>
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<tr>
<td>Q21930</td>
<td>N°(QSY° 7)-N°(9-fluorenylmethoxycarbonyl)-L-lysine (a-FMOC-ε-QSY° 7-L-lysine)</td>
<td>5 mg</td>
</tr>
<tr>
<td>Q20131</td>
<td>QSY° 9 carboxylic acid, succinimidyl ester</td>
<td>5 mg</td>
</tr>
<tr>
<td>Q20132</td>
<td>QSY° 21 carboxylic acid, succinimidyl ester</td>
<td>5 mg</td>
</tr>
<tr>
<td>Q20133</td>
<td>QSY° 35 acetic acid, succinimidyl ester</td>
<td>5 mg</td>
</tr>
<tr>
<td>Q21931</td>
<td>N°(QSY° 35)-N°(9-fluorenylmethoxycarbonyl)-L-alanine (a-FMOC-β-QSY° 35-L-alanine)</td>
<td>5 mg</td>
</tr>
<tr>
<td>Q20348</td>
<td>QSY° 35 iodoacetamide</td>
<td>5 mg</td>
</tr>
</tbody>
</table>