was stained with the Amplex Gold reagent and body, which is included in the kit. Finally, the blot peroxidase conjugate of goat anti–mouse IgG antibody (A-11126), followed by a horseradish bated with a mouse monoclonal anti–

bulin were run on an SDS-polyacrylamide gel and (P-6649) containing decreasing amounts of ples of protein molecular weight standards Gold Western Blot Stain Kit #1 (A-21890). Sam-

Figure 9.35 Immunodetection using the Amplex 

Western Blot Stain Kits and Reagents

The Western blot immunodetection technique provides a powerful method for detecting a protein or proteins of interest on a nitrocellulose or PVDF membrane. The proteins on the blot are typically incubated with a primary antibody against the protein of interest, followed either by an enzyme-labeled secondary antibody or by a biotinylated secondary antibody in conjunction with an enzyme-labeled streptavidin. Finally, presence of the enzyme is detected using chromogenic, fluorogenic or chemiluminescent enzyme substrates. Specific proteins and total proteins are difficult to detect on the same blot using conventional chromogenic stains, which has complicated the assessment of protein transfer efficiency. The signal can also be documented using a laser-based scanner. Scanners using light sources near the excitation maxima for the Amplex Gold peroxidation product (~515 nm) provide the highest sensitivity. The signal-amplification effect of the enzymatic reaction allows detection of as little as 1–3 ng of a protein per band, depending on the antibodies used. The signal is stable indefinitely and can be documented using UV epi-illumination and a Polaroid camera; inexpensive Amplex Gold photographic filter (A-24772, Section 24.3) provides for optimal sensitivity. The signal can also be documented using a laser-based scanner. Scanners using light sources near the excitation maxima for the Amplex Gold peroxidation product (~515 nm) provide the highest sensitivity. The Amplex Gold Western Blot Stain Kits provide the Amplex Gold reagent in combination with either goat anti–mouse IgG antibody, goat anti–rabbit IgG antibody or streptavidin (see Table 9.5). The kits can be used in combination with the SYPRO Ruby protein blot stain (S-11791, Section 9.3) for detecting the total protein profile on the same blot.

Each Amplex Gold Western Blot Stain Kit contains the following reagents, which are sufficient to stain ~20 minigel blots (6 cm × 9 cm):
The Amplex Gold reagent (10 vials)
Solvent for the Amplex Gold reagent
Reaction buffer
The horseradish peroxidase conjugate of goat anti–mouse IgG antibody (in Kit #1, A-21890), goat anti–rabbit IgG antibody (in Kit #2, A-21891) or streptavidin (in Kit #3, A-21892)
A detailed protocol

**Pro-Q Western Blot Stain Kits**

Our Pro-Q Western Blot Stain Kits (Table 9.5) use the fluorogenic substrate DDAO phosphate for simple and rapid detection of an antibody or streptavidin conjugated to alkaline phosphatase (Figure 9.36). DDAO phosphate is a remarkable reagent that provides very rapid and highly sensitive fluorescence detection of alkaline phosphatase conjugates. Alkaline phosphatase rapidly converts DDAO phosphate to the long-wavelength, red-fluorescent product, DDAO (Figure 9.37, Figure 10.6). The signal-amplification effect of the enzymatic reaction allows detection of as little as 1–3 ng of a protein per band, depending on the antibodies used. The sensitivity rivals that of chemiluminescence-based techniques, but because it results in a stable fluorescent product, there is no need to perform the reactions in a darkroom or to incubate the blots with X-ray film. Furthermore, the fluorescent signals, unlike transient chemiluminescent signals, can be imaged several times and are stable indefinitely on dried blots.

Our Pro-Q Western Blot Stain Kits include:

- The DDAO phosphate substrate with an appropriate solvent
- An alkaline phosphatase conjugate of either goat anti–mouse IgG antibody (in Kit #1, P-21863 and Kit #2, P-21860), goat anti–rabbit IgG antibody (in Kit #3, P-21864 and Kit #4, P-21861) or streptavidin (in Kit #5, P-21865 and Kit #6, P-21862)
- Detailed protocols for total and specific protein detection

Kits #2, #4 and #6 also include the SYPRO Ruby protein blot stain for highly sensitive detection of total protein on the blot before immunostaining, as described in detail in Section 9.3. Much more sensitive than Ponceau S, Amido black or Coomassie brilliant

<table>
<thead>
<tr>
<th>Kit</th>
<th>Immunostaining Technique*</th>
<th>Total Protein Detection Technique*</th>
<th>Secondary Detection Conjugate</th>
<th>Cat # of Kit</th>
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<tr>
<td>DyeChrome Western Blot Stain Kits #1, #2 and #3</td>
<td>DDAO phosphate (far-red fluorescence, Abs/Em = 275, 645/660)</td>
<td>BODIPY FL, SE (green fluorescence, Abs/Em = 365, 505/515)</td>
<td>Goat anti–mouse IgG–AP</td>
<td>D-21881</td>
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<td>Streptavidin–AP</td>
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<td>DyeChrome Western Blot Stain Kits #4, #5 and #6</td>
<td>ELF 39 phosphate (green fluorescence, Abs/Em = 345/495)</td>
<td>BODIPY TR, SE (red-orange fluorescence, Abs/Em = 300, 590/615)</td>
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<td>DyeChrome Double Western Blot Stain Kit</td>
<td>Amplex Gold (yellow fluorescence, Abs/Em = 515/535)</td>
<td>MDPF (blue fluorescence, Abs/Em = 385/480)</td>
<td>Goat anti–mouse IgG–AP, Goat anti–mouse IgG–HRP</td>
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<td>Streptavidin–HRP</td>
<td>A-21892</td>
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<td>DDAO phosphate (far-red fluorescence, Abs/Em = 275, 645/660)</td>
<td>SYPRO Ruby protein blot stain (red-orange fluorescence, Abs/Em = 280, 450/618)</td>
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<td>Streptavidin–AP</td>
<td>P-21865</td>
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* Absorption (Abs) and emission (Em) maxima, in nm. SE = succinimidyl ester. AP = alkaline phosphatase. HRP = horseradish peroxidase.
blue, and fully compatible with immunodetection techniques, this brilliant red-orange–fluorescent stain makes it easy to routinely obtain valuable information about the total protein complement of the sample. The fluorescent signal of both stains can be visualized using either UV epi-illumination or a laser-based scanner.

Each Pro-Q Western Blot Stain Kit contains sufficient materials to stain approximately ten to twenty 8 cm × 10 cm minigel blots.

**DyeChrome Western Blot Stain Kits**

Our DyeChrome Western Blot Stain Kits (Table 9.5) use a fluorogenic alkaline phosphatase conjugate of a secondary antibody or streptavidin and a fluorogenic alkaline phosphatase substrate for immunodetection of specific proteins in combination with an amine-reactive BODIPY dye to detect all proteins on a blot in a contrasting fluorescent color.1,2 (Figure 9.38).

The DyeChrome Western Blot Stain Kits include a novel method of staining total proteins on blots using our proprietary amine-reactive BODIPY dye. The reactive dye forms a permanent covalent bond with proteins that lasts through subsequent immunostaining.1,2 This staining technique makes it possible to perform simultaneous two-color labeling, with both total protein and immunostained proteins visible at the same time on the same blot (Figure 9.39, Figure 9.40). BODIPY dye–based staining is rapid, simple and highly sensitive — a combination of traits not found in conventional chromogenic dye–based protein stains. Staining with the amine-reactive BODIPY dyes allows the detection of as little as 4 ng of a protein per band in about an hour, with a linear dynamic range of almost two orders of magnitude (Figure 9.41).

We offer two colors of BODIPY total protein blot stains: the green-fluorescent BODIPY FL-X dye, used in combination with DDAO phosphate (Figure 9.40), which produces a red-fluorescent hydrolysis product, and the red-fluorescent BODIPY TR-X dye, used in combination with ELF 39 phosphate (Figure 9.39, Figure 9.42), which produces a green-fluorescent hydrolysis product. The BODIPY dyes can be visualized using UV illumination or a laser-based scanner. The fluorescence signals from the two stains in each kit show very little spectral overlap (Figure 9.43, Figure 9.44) and can be viewed simultaneously and documented separately using the DyeChrome Red/Green Photographic Filter Set (D-24771, Section 24.4).

Note that because reaction of the dye covalently modifies the protein at random locations, staining by the amine-reactive BODIPY dye may complicate or preclude subsequent analysis by mass spectrometry or microsequencing.

DyeChrome Kits #1, #2 and #3 use the fluorogenic alkaline phosphatase substrate DDAO phosphate, which is rapidly converted to the red-fluorescent product DDAO in the presence of alkaline phosphatase. As little as 1–3 ng of protein per band can be detected with this substrate, depending on the primary antibodies used. To counterstain the entire protein complement on the blot, the kits use BODIPY FL-X succinimidyl ester to stain the proteins with a bright green fluorescence. The fluorescence signals from both stains can be visualized using either UV epi-illumination or visible excitation with a laser-based scanner.

DyeChrome Kits #1, #2 and #3 include:

- DDAO phosphate, with an appropriate solvent
- An alkaline phosphatase conjugate of goat anti–mouse IgG antibody (in Kit #1, D-21881), goat anti–rabbit IgG antibody (in Kit #2, D-21882) or streptavidin (in Kit #3, D-21883)
- BODIPY FL-X succinimidyl ester (10 vials) and an appropriate solvent
- A detailed protocol

DyeChrome Kits #4, #5 and #6 use our proprietary ELF 39 phosphate (Figure 9.42), a novel fluorogenic substrate for alkaline phosphatase that rapidly forms a bright green-fluorescent precipitate at the site of enzyme activity. Sensitive and simple to use, this dye permits the detection of as little as 4–8 ng of a protein per band, depending on the primary antibodies used. The fluorescent signal can be visualized using UV epi-illumination and can be easily separated from that of the red-fluorescent...
Figure 9.39 Protein detection with the DyeChrome Western Blot Stain Kit #4 (D-21884). Samples of protein molecular weight standards (P-6649) containing decreasing amounts of α-tubulin were run on an SDS-polyacrylamide gel and blotted onto a PVDF membrane. After electrophoresis, the blot was stained with BODIPY TR-X, succinimidyl ester (red signal), to detect total protein. After staining, the blot was incubated with a mouse monoclonal anti-α-tubulin antibody (A-11126), followed by an alkaline phosphatase conjugate of goat anti-mouse IgG antibody, which is included in the kit. Finally, the blot was stained with ELF 39 phosphate (green signal) to detect the alkaline phosphatase enzyme. The signal was visualized under UV epi-illumination. The two fluorescent signals were captured separately, using the DyeChrome Red/Green Photographic Filter Set (D-24771), and the two resulting digital images were overlaid using Adobe Photoshop software.

Figure 9.40 Protein detection with the DyeChrome Western Blot Stain Kit #1 (D-21881). Proteins from a rat fibroblast lysate were separated by 2-D gel electrophoresis and blotted onto a PVDF membrane. The proteins are acidic to basic (left to right) and high to low molecular weight (top to bottom). After electrophoresis, the blot was stained with BODIPY FL-X succinimidyl ester (green) to detect total protein. The blot was then incubated with an anti-α-tubulin antibody (A-11126), followed by the alkaline phosphatase conjugate of goat anti-mouse IgG antibody, which is included in the kit. Finally, the blot was stained with DDAO phosphate (red). The fluorescent signals were visualized using UV epi-illumination. The signals were documented separately, using the DyeChrome Red/Green Photographic Filter Set (D-24771) (A and B), and the resulting images overlaid (C).

Figure 9.41 Linear dynamic range of detection for BODIPY FL-X succinimidyl ester, used as a blot stain. A twofold dilution series of molecular weight markers (P-6649) was loaded onto a gel, electrophoresed and electroblotted to a PVDF membrane. The proteins on the blot were then stained with BODIPY FL-X succinimidyl ester, as described for the DyeChrome Western Blot Stain Kits #1, #2 and #3 (D-21881, D-21882, D-21883). The fluorescence intensity for one of the proteins (carbonic anhydrase) was measured and plotted against the amount of protein loaded in the lane. The result shows a linear dynamic range from 4 ng to 125 ng.

Figure 9.42 Structure of ELF 39 phosphate.
BODIPY TR-X total protein stain included in the kits as a contrasting fluorescent total protein stain. BODIPY TR-X staining can be visualized using either UV epi-illumination or visible excitation with a laser-based scanner. Our DyeChrome Red/Green Photographic Filter Set (D-24771, Section 24.4), which contains two specially selected gelatin filters, is recommended for photography of the dichromatic staining using Polaroid 667 black and white print film.

DyeChrome Kits #4, #5 and #6 include:

- ELF 39 phosphate, with an appropriate solvent
- An alkaline phosphatase conjugate of goat anti–mouse IgG antibody (in Kit #4, D-21884), goat anti–rabbit IgG antibody (in Kit #5, D-21885) or streptavidin (in Kit #6, D-21886)
- BODIPY TR-X succinimidyl ester (10 vials) and an appropriate solvent
- A detailed protocol

**Chemiluminescent Protein Detection on Western Blots**

Chemiluminescent enzyme substrates generally provide the most sensitive and background-free method for detecting specific proteins on Western blots. Molecular Probes offers the BOLD APB chemiluminescent substrate (B-21901) for detection of alkaline phosphatase conjugates on PVDF or nitrocellulose membranes. Developed at Serologicals Corp., this substrate is based on a 1,2-dioxetane molecule that emits bright chemiluminescence upon reaction with alkaline phosphatase. The BOLD APB chemiluminescent substrate is provided as a ready-to-use solution that requires no mixing, making it extremely easy to use — there is no need to worry about special blockers or enhancers that are required for other chemiluminescent substrates. The BOLD APB chemiluminescent substrate has several important features:

- The sensitivity of the BOLD APB substrate is up to 10 times greater than the sensitivity offered by alternative chemiluminescent alkaline phosphatase substrates on PVDF membranes and twofold higher on nitrocellulose membranes.
- The signal-to-noise ratio of chemiluminescence is exceptionally high, allowing for sensitivity potentially several times that of most fluorescence techniques.
- The BOLD APB chemiluminescent substrate emits a strong signal that increases in intensity for two hours and remains approximately constant for at least six more hours, allowing plenty of time for the multiple exposures for optimizing detection sensitivity.
- The BOLD APB chemiluminescent substrate has a five-log dynamic range standard curve.
- The BOLD APB chemiluminescent substrate is provided as a ready-to-use solution.
- Shelf-life of the BOLD APB chemiluminescent substrate is at least one year, when stored at 4°C.

Although the nature of chemiluminescence precludes the simultaneous detection of multiple colors on the same blot, immunodetection by the BOLD APB chemiluminescent substrate can be paired with sequential staining by the SYPRO Ruby protein blot stain (S-11791) for detection of the entire protein profile on the blot (Figure 9.45). In contrast to fluorescent reagents, chemiluminescent reagents do not require an excitation light source; the energy from a chemical reaction generates light. The chemiluminescent signal can be detected by directly exposing the blot to X-ray film or by using a scanning instrument designed for chemiluminescence.

The BOLD APB chemiluminescent substrate is supplied in a 25 mL ready-to-use solution, which is sufficient to stain 25 minigel blots.

**Chromogenic Protein Detection on Western Blots**

Western blotting techniques have conventionally used chromogenic enzyme substrates for detection of specific proteins. Substrates for alkaline phosphatase (AP), horseradish peroxidase (HRP), β-galactosidase or horseradish peroxidase have all been used (Section 7.3, Section 7.6). Conventional chromogenic substrates include:
• For alkaline phosphatase conjugates: the combination of NBT (nitro blue tetrazolium, N-6495) and BCIP (5-bromo-4-chloro-3-indolyl phosphate, B-6492), also available in our NBT/BCIP Reagent Kit (N-6547), yields a dark-blue precipitate at the site of enzyme activity (Figure 9.46).

• For β-galactosidase conjugates: 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal, B-1690, B-22015; Section 10.2) yields a turquoise-colored precipitate at the site of enzyme activity.

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**Immunoreagents and Labeled Avidins for Use in Western Blot Detection**

**Fluorescent Avidin Conjugates**

Molecular Probes prepares NeutrAvidin biotin-binding protein and streptavidin labeled with a vast assortment of fluorescent dyes (Section 7.6, Table 7.17), as well as fluorescent microspheres conjugated to streptavidin (Section 6.5). All of these reagents can be used in combination with biotinylated probes for detection of proteins. Although typically not as sensitive as enzyme-amplified techniques, fluorescent avidins are easy to use and permit multicolor detection of targets.

**Primary Antibodies**

Western blotting relies on immunostaining with antibodies to specific proteins. Molecular Probes has available a variety of primary antibodies that are useful for detecting specific proteins on blotting membranes. These include antibodies directed against:

- Cytochrome oxidase (COX) subunits (Section 12.2)
- Other mitochondrial proteins (Section 12.2)
- Intermediate filament proteins (Section 11.2)
- β-Tubulin (Section 11.2)
- T cell differentiation markers (Section 11.2)
- Cell-cycle proteins (Section 15.4)
- Matrix metalloproteinases (Section 10.4)
- 5-Bromo-2′-deoxyuridine (Section 15.4)
- Second messenger compounds (Section 18.4)
- Neuronal markers (Section 7.5)
- Human transferrin receptor (Section 7.5)

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**Figure 9.45** Immunodetection on a Western blot with the BOLD APB chemiluminescent substrate. Samples of protein molecular weight standards (P-6649) containing decreasing amounts of α-tubulin were run on an SDS-polyacrylamide gel and blotted onto a PVDF membrane. After electrophoresis, the blot was stained with SYPRO Ruby protein blot stain (S-11791) to detect total protein. After documentation of the total protein stain (top), the blot was incubated with a mouse monoclonal anti-α-tubulin antibody (A-11126), followed by an alkaline phosphatase conjugate of goat anti-mouse IgG antibody (G-21060). Finally, the blot was stained with the BOLD APB chemiluminescent substrate (B-21901) to detect the alkaline phosphatase enzyme. The chemiluminescent signal was visualized using a scanner in chemiluminescence detection mode.

**Figure 9.46** Principle of enzyme-linked detection using the reagents in our NBT/BCIP Reagent Kit (N-6547). Phosphatase hydrolysis of BCIP is coupled to reduction of NBT, yielding a formazan and an indigo dye that together form a black-purple–colored precipitate.
Figure 9.47 Detection of HA-fusion proteins using anti-HA antibody. Six proteins, each fused to the HA domain, were electrophoresed through a 13% polyacrylamide gel and blotted onto a PVDF membrane. The blot was incubated with the Alexa Fluor 488 conjugate of anti-HA antibody (A-21287), followed by rabbit anti–Alexa Fluor 488 antibody (A-11094). The antibody complex was then detected using the Amplex Gold Western Blot Stain Kit #2 (A-21891).

Molecular Probes also provides antibodies against epitope and protein tags for detecting appropriately tagged recombinant proteins:

- c-myc tag (A-21280, A-21281; Section 7.5)
- Glutathione S-transferase (GST) (A-5800, A-11131; Section 7.5)
- β-Galactosidase (A-11132, Section 10.2)
- β-Glucuronidase (GUS) (A-5790, Section 10.2)
- Oligohistidine fusion proteins (P-21315; see the description later in this section)
- Hemagglutinin (HA)-tag (A-21287, A-21288; Section 7.5; Figure 9.47)

Finally, our anti-dye, anti-biotin, anti-DNP and anti-nitrotyrosine (Figure 19.20) antibodies can be employed for the selective detection of primary or secondary proteins labeled with fluorescent dyes, biotin, DSB-X biotin or the DNP or o-nitrophenol haptens (Section 7.4, Table 7.13); Section 4.2 describes our recommended reagents for labeling proteins and nucleic acids with biotin (Table 4.1) and haptens (Table 4.2).

**Multiplex Western Blots: Detecting Multiple Protein Targets Simultaneously**

**DyeChrome Double Western Blot Stain Kit**

The DyeChrome Double Western Blot Stain Kit (D-21887) is our first detection kit for Multiplexed Proteomics on Western blots that permits the use of two different enzyme-conjugated antibodies and a general protein stain for the simultaneous **trichromatic** detection of multiple targets on the same blot, (Figure 9.48). The reagent combinations in this kit are:

- A horseradish peroxidase (HRP) conjugate of goat anti–rabbit IgG antibody and the Amplex Gold reagent for yellow-fluorescent detection of a rabbit antibody to a specific protein or proteins
- An alkaline phosphatase conjugate of goat anti–mouse IgG antibody and DDAO phosphate for far red-fluorescent detection of a mouse antibody to a specific protein or proteins
- MDPF (2-methoxy-2,4-diphenyl-3(2H)-furanone) for blue-fluorescent detection of the total protein profile
- Appropriate solvents and buffers for the enzymatic reactions
- A detailed protocol

Each DyeChrome Double Western Blot Stain Kit contains sufficient materials to stain ~20 minigel blots (6 cm x 9 cm). The two antigens are developed and detected simultaneously; staining is stable indefinitely on dried blots.

**Molecular Probes’ Zenon Technology for Efficient Staining of Western Blots**

Primary antibodies on Western blots have usually been detected indirectly with an enzyme-conjugated secondary antibody or with avidin–biotin technology, as described above and in Section 7.3 and Section 7.6. However, our exclusive Zenon One technology, which is described in detail in Section 7.2, makes it possible to directly label any mouse or rat IgG1 monoclonal antibody with an extensive assortment of dyes or with enzymes, including alkaline phosphatase and HRP (Table 7.1, Figure 7.32). This direct labeling technology makes it possible to use two or more mouse IgG1 primary antibodies for detection of different specific proteins on the same blot. A particularly significant advantage of the Zenon technology is its utility for forming useful enzyme complexes starting with even submicrogram quantities of the primary antibody. The primary antibodies can be labeled with fluorescent dyes, for more abundant targets (Figure 12.32), or with enzymes to allow

Figure 9.49 Multiplex detection of two different antibodies on the same blot using fluorophore-labeled secondary antibodies. Cell lysates from nonstimulated (left band) or EGF-stimulated (right band) A431 cells were electrophoresed in an SDS-polyacrylamide gel and blotted. The blot was incubated with primary antibodies against ERK1 and ERK2, p44/42 MAP kinases. Total ERK protein was detected using rabbit anti–ERK IgG antibody followed by anti–rabbit IgG antibody labeled with IRDye 800 (green, Licor). Tyrosine-phosphorylated ERK was detected using mouse anti–phospho-ERK IgG antibody, followed by Alexa Fluor 580 anti–mouse IgG antibody (red, A-21057). The blots were imaged using the Odyssey Infrared Imaging System (Licor). Each signal is shown separately (top and middle) and viewed simultaneously on digitally overlaid images (bottom).
amplification of the signal with fluorogenic, chromogenic or chemiluminescent enzyme substrates. Depending on the primary antibodies used, it is possible to detect multiple targets with little to no crossreactivity. The Zenon One labeling is simple and very rapid — the reaction is complete in just minutes after the primary antibody and Zenon One reagent are mixed together and no purification steps are required before use.

The Zenon One Alkaline Phosphatase Mouse IgG1 Labeling Kit (Z-25050) provides the reagents and instructions for the rapid and quantitative preparation of mouse IgG1 antibodies labeled with alkaline phosphatase, which can then be used in conjunction with the BOLD APB chemiluminescent substrate or with either our ELF 39 phosphate or our DDAO phosphate fluorogenic phosphatase substrates to detect proteins on Western blots. The Zenon One Horseradish Peroxidase Mouse IgG1, Labeling Kit (Z-25054) can similarly be used to prepare HRP-labeled complexes of mouse monoclonal antibodies for detection with the Amplex Gold peroxidase substrate. Zenon complexes can also be prepared from multiple mouse primary antibodies by using dyes in our Zenon One Kits that have contrasting fluorescence.

Staining with Two Different Labeled Primary Antibodies

To use multiple antibodies on the same blot, the secondary or primary antibodies may also be labeled directly with amine-reactive dyes, such as the succinimidyl esters described in Chapter 1. Direct labeling usually provides somewhat lower sensitivity than indirect labeling using enzymatic substrates because the enzymatic substrates can greatly amplify the signal. However, for abundant proteins, direct labeling provides a more streamlined method for staining the blot with multiple antibodies. The limit on the number of colors that can be used together depends only on the compatibility of the antibodies used and the ability of the instrumentation to separate the signals from the fluorescent dyes used (Figure 9.49).

Fluorescence-Based Technologies for the Detection of Oligohistidine Fusion Proteins

The oligohistidine domain is a Ni$^{2+}$-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. Molecular Probes has developed technologies that make it possible to quickly and easily identify oligohistidine fusion proteins in gels or on blots.

Pro-Q Sapphire Oligohistidine Gel Stains

The Pro-Q Sapphire 365 and Pro-Q Sapphire 488 oligohistidine gel stains (P-21876, P-21877) provide a simple method for the detection of oligohistidine fusion proteins directly in an SDS-polyacrylamide gel (Figure 9.50, Figure 9.51), eliminating the need to blot the protein to a membrane. These proprietary reagents each comprise a state-of-the-art fluorescent dye conjugated to a nitrilotriacetic acid (NTA) moiety. The staining procedure is very simple — simply fix the gel and incubate it with the stain. The NTA moiety chelates Ni$^{2+}$ bound by the oligohistidine domain, resulting in optimal staining in just 45 minutes. Note that because the NTA is negatively charged, there may also be some weak crossreactivity with highly basic proteins. The Pro-Q Sapphire 365 oligohistidine gel stain (P-21876) can be viewed using 365 nm UV illumination and the SYPRO photographic filter (S-6656, Section 24.4, Figure 24.51) and has a sensitivity limit of ~30 ng/band of an oligohistidine fusion protein. The Pro-Q Sapphire 488 oligohistidine gel stain (P-21877) can be viewed using visible light with wavelengths near its 510 nm excitation maximum and has a sensitivity limit of ~30 ng/band. These limits of sensitivity were determined using a hexahistidine–urate oxidase fusion protein; other fusion proteins we have tested show levels of sensitivity between 60 and 100 ng per band, suggesting that the protein environment may have an effect on the ability of NTA-based compounds to bind to oligohistidine domains. After documenting the oligohistidine signal, the total protein profile of the gel can be visualized using the SYPRO Ruby protein gel stain (Section 9.3, Figure 9.50).
Figure 9.50 Twofold dilutions of three different *Escherichia coli* lysates, each expressing a recombining oligohistidine fusion protein, were run on an SDS-polyacrylamide gel (lanes 4–6, 7–9 and 10–12). Lane 1 contains molecular weight standards, lane 2 contains a 6xHis protein ladder (QIAGEN), lane 3 contains a control lysate with hexahistidine-tagged urate oxidase (Pierce), and lane 13 contains purified BSA. After electrophoresis, the gel was stained using the Pro-Q Sapphire 488 oligohistidine gel stain (left). After documentation of the signal, the gel was stained with the SYPRO Ruby protein gel stain (S-12000, S-12001, S-21900; bottom).

Figure 9.51 Staining of an oligohistidine fusion protein with the Pro-Q Sapphire 488 oligohistidine gel stain (P-21877). Twofold dilutions of an *Escherichia coli* lysate containing overexpressed oligomycin sensitivity–confering protein (OSCP) fused with an oligohistidine domain were run on an SDS-polyacrylamide gel. After electrophoresis, the gel was stained using the Pro-Q Sapphire 488 oligohistidine gel stain (left). After documentation of the oligohistidine signal, the gel was stained for total protein (right) using the SYPRO Ruby protein gel stain (S-12000, S-12001, S-21900).

**Pro-Q Oligohistidine Blot Stain Kits**

The Pro-Q Oligohistidine Blot Stain Kits provide a simple, fast and sensitive method for the detection of oligohistidine fusion proteins on PVDF membranes. The staining technique uses biotin-X nitrotriacetic acid (biotin-X NTA, Figure 4.6), which chelates Ni^{2+}. The blot is incubated with a complex of biotin-X NTA, Ni^{2+} and streptavidin–alkaline phosphatase. Within 20 minutes, the complex binds to oligohistidine fusion proteins. The complex is then detected using a fluorogenic alkaline phosphatase substrate — either DDAO phosphate (in Kit #1; P-21878, Figure 9.52), which produces a red-fluorescent product, or ELF 39 phosphate (in Kit #2; P-21879, Figure 9.42), which produces a green-fluorescent product. Both substrates (described earlier in the section about Western blotting technologies) provide very rapid and sensitive detection of the alkaline phosphatase conjugates, making it possible to detect as little as ~16 ng/band in less than 90 minutes after blotting, depending on the particular fusion protein. The sensitivity of these fluorogenic substrates rivals that of chemiluminescence detection. However, because the fluorescent products are chemically stable, there is no need to perform the reaction in a darkroom or to incubate the blot with X-ray film. Furthermore, the fluorescent signal, unlike transient chemiluminescent signals, can be imaged several times and is stable indefinitely on dried blots. The biotin-X NTA and DDAO can be removed from the blot for restaining with another detection method; the ELF 39 stain, however, is permanent. Biotin-X NTA is also available separately (B-11790).

**Penta·His Antibody**

Developed by QIAGEN, the Penta·His mouse IgG1 monoclonal antibody (P-21315) 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 blots in combination with our Western Blot Stain Kits (Figure 9.53; see the description of our Pro-Q, Amplex Gold and DyeChrome Western Blot Stain Kits, earlier in this section). Alternatively, the mouse IgG1 isotype Penta·His antibody can be rapidly and quantitatively complexed with any of our fluorescent dye– or enzyme-conjugated Zenon One reagents (Table 7.1) for detecting oligohistidine fusion proteins by almost any assay scheme. The Penta·His antibody is also useful for immunoprecipitation, ELISA assays, and immunohistochemistry.

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

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

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

Our Pro-Q Emerald 300 and Pro-Q Emerald 488 Glycoprotein Stain Kits (Table 9.6) provide the most advanced reagents known for detecting glycoproteins in gels and on blots. These stains are easier to use and more sensitive than any other glycoprotein staining technique (Table 9.7). The Pro-Q Emerald glycoprotein stains react with periodate-oxidized carbohydrate groups, creating a bright green-fluorescent signal on glycoproteins (Figure 9.54). The staining procedure requires only three steps: fixation, oxidation and staining — no reduction step is required (Figure 9.55). 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-fold more sensitive than the standard periodic acid–Schiff base method using acidic fuchsin dye. Blot staining is not quite as sensitive (detecting 2–
18 ng of a glycoprotein per band) 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 excitation maximum. The Pro-Q Emerald dye is also used as the detection reagent in our Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (P-20495), which is described in Section 3.2 (Figure 3.18, Figure 3.19, Figure 3.20).

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 9.56, Figure 9.57, Figure 9.58). For this purpose, we offer the Pro-Q Emerald Glycoprotein Gel Stain Kits (P-21855, P-21873) and the Pro-Q Emerald Glycoprotein Blot Stain Kits (P-21856, P-21874). These kits include our patented SYPRO Ruby protein gel stain or blot stain (described in Section 9.3) for detection of total proteins. The

Table 9.6 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 #</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>P-21855</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blot Stain Kit (includes SYPRO Ruby protein blot stain *)</td>
<td>P-21856</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gel and Blot Stain Kit (does not include a total protein stain)</td>
<td>P-21857</td>
</tr>
<tr>
<td>Pro-Q Emerald 488 Glycoprotein Stain Kits</td>
<td>Pro-Q Emerald 488 stain, Ex/Em = 510/520 nm</td>
<td>Gel Stain Kit (includes SYPRO Ruby protein gel stain *)</td>
<td>P-21873</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blot Stain Kit (includes SYPRO Ruby protein blot stain *)</td>
<td>P-21874</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gel and Blot Stain Kit (does not include a total protein stain)</td>
<td>P-21875</td>
</tr>
</tbody>
</table>

* See Section 9.3 for a description of the SYPRO Ruby protein stains.

Table 9.7 Comparison of various commercially available glycoprotein stain kits.

<table>
<thead>
<tr>
<th>Product</th>
<th>Detection Technology *</th>
<th>Staining Time †</th>
<th>Sensitivity Limits ‡</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-Q Emerald 300 Glycoprotein Stain Kits (Molecular Probes)</td>
<td>Pro-Q Emerald 300 stain</td>
<td>2.5 hours 6 steps</td>
<td>1–2 ng/band (gel kit) 2–18 ng/band (blot kit)</td>
<td>• Signal stable indefinitely § • View with 300 nm illumination</td>
</tr>
<tr>
<td>Pro-Q Emerald 488 Glycoprotein Stain Kits (Molecular Probes)</td>
<td>Pro-Q Emerald 488 stain</td>
<td>2.5 hours 6 steps</td>
<td>4 ng/band (gel kit) 4 ng/band (blot kit)</td>
<td>• Signal stable indefinitely § • View with visible light (450–510 nm)</td>
</tr>
<tr>
<td>Pro-Q Fuchsia Glycoprotein Stain Kits (Molecular Probes)</td>
<td>Acid-Schiff base chemistry with acid fuchsin dye (PAS method)</td>
<td>2.5 hours 7 steps</td>
<td>75–150 ng/band (gel kit) 20–75 ng/band (blot kit)</td>
<td>• Permanent signal</td>
</tr>
<tr>
<td>ECL Glycoprotein Detection (Amersham-Pharmacia)</td>
<td>Biotin hydrazide reaction followed by streptavidin–HRP followed by ECL detection reagents</td>
<td>6 hours 11 steps</td>
<td>18–150 ng/band (blots)</td>
<td>• Transient signal fades quickly</td>
</tr>
<tr>
<td>DIG Glycan Detection Kit (Roche)</td>
<td>Digoxigenin hydrazide reaction followed by anti-digoxigenin–AP followed by NBT/BCIP</td>
<td>6 hours 11 steps</td>
<td>2–37 ng/band (blots)</td>
<td>• Permanent signal • Cross-reaction with carbonic anhydrase</td>
</tr>
<tr>
<td>GlycoTrack Detection Kit (Glyko)</td>
<td>Biotin hydrazide reaction followed by streptavidin–AP followed by NBT/BCIP</td>
<td>6 hours 11 steps</td>
<td>2–37 ng/band (blots)</td>
<td>• Permanent signal • Cross-reaction with carbonic anhydrase</td>
</tr>
</tbody>
</table>

* All detection procedures begin with periodate oxidation of carbohydrates. † Includes all staining and wash steps, but does not include time for blotting required in the blot detection kits. ‡ Sensitivities were measured for three glycoproteins of differing carbohydrate components: 1-acidic glycoprotein (40% carbohydrate), glucose oxidase (12% carbohydrate) and avidin (7% carbohydrate). The ranges reported represent differences in detection sensitivity for the different glycoproteins. § If stored protected from light. HRP = Horseradish peroxidase conjugate. AP = Alkaline phosphatase conjugate. NBT/BCIP = Colorimetric detection of alkaline phosphatase using nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.
easy-to-use SYPRO Ruby protein stains 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. The total protein stain makes 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. SYPRO Ruby protein blot stain is additionally useful for assessing the efficiency of protein transfer to a blot (Figure 9.58), which is especially important when working with glycoproteins, because they often transfer poorly to blotting membranes. Proteins show red-orange–fluorescent staining 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 Detection Kits also include our exclusive CandyCane molecular weight standards, a mixture of glycosylated and nonglycosylated proteins that, when separated by electrophoresis, provide alternating positive and negative controls (Figure 9.59). The CandyCane molecular weight standards are also available separately (C-21852). The Pro-Q Emerald Glycoprotein Gel Stain Kits contain:

- The Pro-Q Emerald glycoprotein stain
- A staining buffer
- Periodic acid, an oxidizing reagent
- SYPRO Ruby protein gel stain (in Kits P-21855 and P-21873) or SYPRO Ruby protein blot stain (in Kits P-21856 and P-21874)
- CandyCane glycoprotein molecular weight standards
- Detailed protocols

Each kit provides sufficient materials to stain approximately ten 8 cm × 10 cm gels or blots.

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

The Pro-Q Fuchsia Glycoprotein Stain Kits (P-21850, P-21851) provide another useful method for differentially staining glycosylated and nonglycosylated proteins on SDS-polyacrylamide gels or blotting membranes. Unlike our Pro-Q Emerald 300 Glyco-
protein Stain Kits, in which a fluorometric method is used to detect glycosylated proteins, the Pro-Q Fuchsia Glycoprotein Stain Kits employ a colorimetric method for selective staining of glycoproteins. This method is based on the periodic acid–Schiff (PAS) procedure to detect carbohydrate groups. Upon exposure to periodic acid, glycols present in glycoproteins are oxidized to aldehydes. The Pro-Q Fuchsia reagent, an acidic fuchsin sulfite, then reacts with the aldehydes on the glycoproteins to generate an addition product that produces a characteristic fuchsia-colored stain, which is visible in normal room light (Figure 9.60). This method can detect as little as 25–100 ng of glycoprotein, depending on the nature and the degree of glycosylation of the protein, which may contain one to hundreds of mono- or polysaccharides attached to a single polypeptide chain. This stain detects both N-linked and O-linked oligosaccharides, which are attached covalently through the asparagine residues and serine or threonine residues, respectively. The Pro-Q Fuchsia reagent is much more specific than 1,9-dimethylmethylene blue– or alcian blue–staining procedures, which also detect some acidic nonglycosylated proteins.

As with our Pro-Q Emerald Glycoprotein Stain Kits, the Pro-Q Fuchsia Glycoprotein Stain Kits use SYPRO Ruby protein stains to detect total proteins on gels or blots (Figure 9.60). Proteins show red-orange–fluorescent staining when illuminated with a 300 nm UV light source or a laser scanner with a 473, 488 or 532 nm light source.

The Pro-Q Fuchsia Glycoprotein Gel Stain Kits also include our exclusive CandyCane molecular weight standards, a mixture of glycosylated and nonglycosylated proteins that, when separated by electrophoresis, provide alternating positive and negative controls (Figure 9.60). The CandyCane molecular weight standards are also available separately (C-21852).

Each Pro-Q Fuchsia Glycoprotein Gel Stain Kit (P-21850) and Pro-Q Fuchsia Glycoprotein Blot Stain Kit (P-21851) contains:

- The Pro-Q Fuchsia reagent
- Periodic acid, an oxidizing reagent
- Sodium metabisulfite, a reducing reagent
- SYPRO Ruby protein gel stain (in Kit P-21850) or SYPRO Ruby protein blot stain (in Kit P-21851)
- CandyCane glycoprotein molecular weight standards
- Detailed protocols

Each kit provides sufficient materials to stain approximately ten 8 cm × 10 cm gels or blots.

**Pro-Q Glycoprotein Blot Stain Kits with Lectins**

Our lectin-based Pro-Q Glycoprotein Blot Stain Kits provide high-sensitivity detection of specific sugar residues in glycoproteins (Table 9.8, Figure 9.61). To detect termi-
nal $\alpha$-mannopyranosyl and $\alpha$-glycopyranosyl residues that do not contain tri- or tetraantennary structures, we have developed the Pro-Q Glycoprotein Blot Stain Kits #1 and #2 with Concanavalin A (P-21870, P-21853; Figure 9.62), which employ concanavalin A (Con A) from *Canavalia ensiformis* (Jack bean) seeds. Our Pro-Q Glycoprotein Blot Stain Kits #3 and #4 (P-21871, P-21854) use wheat germ agglutinin (WGA) to detect $N$-acetylglucosamine and $N$-acytelneuraminic acid (sialic acid) residues. Our Pro-Q Glycoprotein Blot Stain Kit #5 (P-21872) is supplied with *Griffonia simplicifolia* lectin II (GS-II), which recognizes terminal nonreducing $N$-acetylglucosamine residues. All of these kits use an alkaline phosphatase conjugate of the lectin as a convenient means of selectively detecting the corresponding glycoproteins on nitrocellulose or PVDF membranes, providing valuable information about the structure of glycoproteins in an experimental sample.

The detection procedure in these kits is similar to that of Western blotting and uses the fluorogenic alkaline phosphatase substrate, DDAO phosphate, for the final detection step. After the proteins are separated on a polyacrylamide gel, they are blotted onto a membrane and incubated with the lectin–alkaline phosphatase conjugate. The alkaline phosphatase enzyme is then detected using DDAO phosphate, which is rapidly converted to the long-wavelength, red-fluorescent product DDAO (Figure 10.6). The enzymatic reaction greatly amplifies the signal, making it possible to detect as little as 15 ng of a glycoprotein, depending on the degree and nature of glycosylation. Sensitivity of the DDAO phosphate–based detection technique rivals ECL chemiluminescence detection, but because DDAO phosphate–based detection produces a stable fluorescent product, there is no need to perform the reaction in a darkroom or to expose the blot to X-ray film. Additionally, unlike transient chemiluminescent signals, the red-fluorescent DDAO signal can be imaged several times and is stable indefinitely on dried blots. The fluorescent signal can be visualized using UV epi-illumination or a laser-based scanner.

As with our Pro-Q Fuchsia Glycoprotein Stain Kit described above, the Pro-Q Glycoprotein Blot Stain Kit #2 with Concanavalin A (P-21853) and Pro-Q Glycoprotein Blot Stain Kit #4 with wheat germ agglutinin (WGA) (P-21854) also include the SYPRO Ruby protein gel stain for ultrasensitive fluorescent detection of total protein. When used prior to glycoprotein detection, SYPRO Ruby blot stain makes it possible to assess the level of protein transfer to the blot, to compare stained proteins with molecular weight markers and to identify contaminating proteins in the sample.

These kits also include our exclusive CandyCane molecular weight standards (Figure 9.59), which includes glycosylated (three that are recognized by Con A and four that are recognized by WGA) and nonglycosylated proteins. The CandyCane molecular weight standards are also available separately (C-21852).

The Pro-Q Glycoprotein Blot Stain Kits include:

- An alkaline phosphatase conjugate of Con A (in Kits P-21853 and P-21870), WGA (in Kits P-21854 and P-21871) or GS-II (in Kit P-21872)
- DDAO phosphate
- Dimethylformamide (DMF)
- CandyCane glycoprotein molecular weight standards

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Detected Structure *</th>
<th>Cat # †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concanavalin A</td>
<td>$\alpha$-Mannosyl and $\alpha$-glucosyl residues</td>
<td>P-21853, P-21870</td>
</tr>
<tr>
<td></td>
<td>Core pentasaccharide of $N$-linked glycans</td>
<td>P-21853, P-21870</td>
</tr>
<tr>
<td></td>
<td>Does not detect tri- or tetraantennary structures of complex $N$-linked glycans</td>
<td>P-21853, P-21870</td>
</tr>
<tr>
<td>Wheat germ agglutinin</td>
<td>$\text{Sialic acid and } N\text{-acetylglucosaminyl residues}$</td>
<td>P-21854, P-21871</td>
</tr>
<tr>
<td></td>
<td>Does not detect tetraantennary structures of complex $N$-linked glycans</td>
<td>P-21854, P-21871</td>
</tr>
<tr>
<td>GS-II</td>
<td>Terminal, non-reducing $N$-acetylglucosaminyl residues</td>
<td>P-21872</td>
</tr>
</tbody>
</table>

* More complete information on lectin binding specificities is available at http://plab.ku.dk/tcbh/Lectins12/DiVirgilio/paper.htm. † Pro-Q Glycoprotein Stain Kits with lectins contain sufficient reagents to stain 10–20 minigel blots.
• SYPRO Ruby protein blot stain (in Kits P-21853 and P-21854 only)
• Detailed protocols (with Con A; with WGA; with GS-II)

Each kit provides sufficient materials to stain approximately ten to twenty 8 cm × 10 cm minigel blots.

CandyCane Glycoprotein Molecular Weight Standards
CandyCane glycoprotein molecular weight standards (C-21852) 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 9.59). 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 Gel Stain Kits (see above).

Specialized Techniques for Detection of Specific Proteins in Gels

Detection of Calcium-Binding Proteins in Gels
The luminescent lanthanide terbium, which is available from Molecular Probes as its chloride salt (Tb⁺⁺ from TbCl₃, T-1247), selectively stains calcium-binding proteins in SDS-polyacrylamide gels.⁹ With some modifications to the staining protocol, these lanthanides can also be used to detect all protein bands.⁹ 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.¹⁰

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**Figure 9.59** Glycosylated and nonglycosylated proteins in the Candy-Cane glycoprotein molecular weight standards (C-21852). 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 (S-12000, S-12001, S-21900) to detect all eight marker proteins. The right lane was stained using the reagents in the Pro-Q Emerald 300 Glycoprotein Gel Stain Kit (P-21855).

**Figure 9.60** Detection of glycoproteins and total protein on an SDS-polyacrylamide gel using the Pro-Q Fuchsia Glycoprotein Gel Stain Kit (P-21850). CandyCane glycoprotein molecular weight standards (C-21852) containing alternating glycosylated and nonglycosylated proteins were electrophoresed through a 13% polyacrylamide gel. After separation, the gel was stained with SYPRO Ruby protein gel stain (S-12000, S-12001, S-21900) to detect all eight marker proteins (left). Subsequently, the gel was stained by the standard periodic acid–Schiff base (PAS) method in the Pro-Q Fuchsia Glycoprotein Gel Stain Kit to detect the glycoproteins α₂-macroglobulin, glucose oxidase, α₁-glycoprotein and avidin (right).

**Figure 9.61** Primary binding sites of lectins in the Pro-Q Glycoprotein Blot Stain Kits.
Detection of Enzymes in Nondenaturing Gels

A wide variety of enzymes have been detected in nondenaturing gels by using various chromogenic substrates, including X-Gal (B-1690, B-22015; Section 10.2), X-GlU (B-1691, Section 10.2) and NBT/BCIP 11 (N-6495, B-6492; Section 10.3). In unpublished experiments, we have shown that our ELF 97 phosphatase substrate (E-6589, Section 10.3) forms a highly fluorescent precipitate at the site of enzymatic activity (either acid or alkaline phosphatase activity) in nondenaturing polyacrylamide gels. We have also demonstrated that our ELF 97 β-glucuronidase substrate (E-6587, Section 10.2) has similar utility for detecting β-glucuronidase in native or SDS-polyacrylamide gels, with a detection limit of less than 5 ng of the enzyme 12 (Figure 10.22). The ELF 97 β-D-glucuronidase substrate can also be used in combination with our SYPRO Tangerine protein gel stain (S-12010, Section 9.3) for detection of the total protein profile in the gel (Figure 9.24). Fluorogenic protease substrates based on the rhodamine 110 dye (Section 10.4, Table 10.2) have been impregnated in filter paper and overlaid on SDS-polyacrylamide gels to detect protease activity.13

Detection of Protein Functional Groups in Gels and on Blots

Several of the low molecular weight, thiol-reactive reagents described in Chapter 2 can potentially be used to selectively detect thiol-containing proteins in gels without appreciable staining of proteins that do not contain thiols. Compounds that may be particularly useful include BODIPY 493/503 methyl bromide and BODIPY 630/650 methyl bromide (B-2103, B-22802; Section 2.2), IANBD amide (D-2004, Section 2.2), monochlorobimane and monobromobimane 14 (M-1381, M-1378; FluoroPure Grade, M-20381; Section 2.3), the coumarin iodoacetamide IDCC (D-20382, Section 2.3) and CellTracker Blue CMAC (C-2110, Section 14.2). These selected compounds are all electrically neutral reagents and thus do not appreciably change the charge or mass of the protein, a feature that may make them useful for derivatizing the thiolated protein prior to separation by isoelectric focusing. Monobromobimane has been used to derivatize thiol-containing proteins prior to separation by isoelectric focusing without the modification having an appreciable effect on the protein’s electrophoretic mobility.15–18 8-Aminonaphthalene-1,3,6-trisulfonic acid (A-350, Section 3.2) has been used to directly stain periodate-oxidized glycoproteins on PVDF membranes.19 Glycoprotein binding to PVDF membranes was selectively enhanced by pre-coating the membrane with wheat germ agglutinin (WGA). Alexa Fluor 350 hydrazide (A-10439, Section 3.2) has been used for similarly for glycoprotein detection both in gels and on blots.8

Detection of Penicillin-Binding Proteins

BOCILLIN FL penicillin and BOCILLIN 650/665 penicillin (B-13233, B-13234) are green- and infrared-fluorescent penicillin analogs, respectively, that bind selectively and with high affinity to penicillin-binding proteins.20,21 When electrophoresed under nonreducing conditions the dye-labeled penicillin-binding proteins are easily visible in the gel 22 (Figure 9.63).

Chemical Labeling of Nascent Proteins

The relatively compact BODIPY FL fluorophore (D-6140, Section 1.4) has been used as a fluorescent reporter group in nascent proteins. This dye was incorporated at the N-terminus
of nascent proteins using an *Escherichia coli* tRNA(fmet) mis-
aminocacylated with a methionine containing a BODIPY FL
fluorophore at its amino group.\textsuperscript{23} Under optimal conditions,
subnanogram quantities of green–fluorescent bands from *in vitro–*
produced fluorescent proteins can be detected by gel electro-
phoresis using a laser-based gel scanner.

**Mitochondrial Protein Extracts**

For researchers seeking a source of mitochondrial protein
standards, Molecular Probes offers human heart mitochondrial proteins for SDS polyacrylamide gel electrophoresis (M-22430) and 2-D gel electrophoresis (M-22431, Section 12.2). These products are complete mitochondrial lysates that have tested negative for hepatitis B and C as well as HIV 1 and 2 in serology tests. The mitochondrial protein standards are useful for compari-
son to new mitochondrial protein preparations in either 1-D or
2-D gels and for testing mitochondrial antibodies.

**Product List — 9.4 Multiplex Proteomics for Detection of Specific Proteins in Gels and on Blots**

<table>
<thead>
<tr>
<th>Cat #</th>
<th>Product Name</th>
<th>Unit Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-24772</td>
<td>Amplex \textsuperscript{®} Gold photographic filter</td>
<td>each</td>
</tr>
<tr>
<td>A-21890</td>
<td>Amplex \textsuperscript{®} Gold Western Blot Stain Kit #1 <em>with goat anti-rabbit IgG</em> <em>&gt;20 minigel blots</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>A-21891</td>
<td>Amplex \textsuperscript{®} Gold Western Blot Stain Kit #2 <em>with goat anti-rabbit IgG</em> <em>&gt;20 minigel blots</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>A-21892</td>
<td>Amplex \textsuperscript{®} Gold Western Blot Stain Kit #3 <em>with streptavidin</em> <em>&gt;20 minigel blots</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>B-11790</td>
<td>biotin-X nitriolactic acid, tripotassium salt (biotin-X NTA)</td>
<td>5 mg</td>
</tr>
<tr>
<td>B-13233</td>
<td>BOCILLIN\textsuperscript{®} FL penicillin, sodium salt</td>
<td>1 mg</td>
</tr>
<tr>
<td>B-13234</td>
<td>BOCILLIN\textsuperscript{®} 650/665 penicillin, sodium salt</td>
<td>1 mg</td>
</tr>
<tr>
<td>B-21901</td>
<td>BOLD\textsuperscript{®} APB chemiluminescent substrate <em>for membrane-based alkaline phosphatase detection</em> <em>&gt;25 minigel blots</em></td>
<td>25 mL</td>
</tr>
<tr>
<td>C-21582</td>
<td>Candyglove\textsuperscript{®} glycoprotein molecular weight standards <em>&gt;200 gel lanes</em></td>
<td>400 µL</td>
</tr>
<tr>
<td>D-21887</td>
<td>DyeChrome\textsuperscript{™} Double Western Blot Stain Kit <em>for mouse IgG, rabbit IgG and total protein detection</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>D-21881</td>
<td>DyeChrome\textsuperscript{™} Western Blot Stain Kit #1 <em>with goat anti-mouse IgG, DDAO phosphate and BODIPY\textsuperscript{®} FL-X, SE</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>D-21882</td>
<td>DyeChrome\textsuperscript{™} Western Blot Stain Kit #2 <em>with goat anti-rabbit IgG, DDAO phosphate and BODIPY\textsuperscript{®} FL-X, SE</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>D-21883</td>
<td>DyeChrome\textsuperscript{™} Western Blot Stain Kit #3 <em>with streptavidin, DDAO phosphate and BODIPY\textsuperscript{®} FL-X, SE</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>D-21884</td>
<td>DyeChrome\textsuperscript{™} Western Blot Stain Kit #4 <em>with goat anti-mouse IgG, ELFS\textsuperscript{®} 39 phosphate and BODIPY\textsuperscript{®} TR-X, SE</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>D-21885</td>
<td>DyeChrome\textsuperscript{™} Western Blot Stain Kit #5 <em>with goat anti-rabbit IgG, ELFS\textsuperscript{®} 39 phosphate and BODIPY\textsuperscript{®} TR-X, SE</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>D-21886</td>
<td>DyeChrome\textsuperscript{™} Western Blot Stain Kit #6 <em>with streptavidin, ELFS\textsuperscript{®} 39 phosphate and BODIPY\textsuperscript{®} TR-X, SE</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>M-22431</td>
<td>mitochondrial proteins (human heart) for 2-D gel electrophoresis <em>2 mg/mL</em></td>
<td>100 µL</td>
</tr>
<tr>
<td>M-22430</td>
<td>mitochondrial proteins (human heart) for SDS-polyacrylamide gel electrophoresis <em>2 mg/mL</em></td>
<td>100 µL</td>
</tr>
<tr>
<td>P-21315</td>
<td>Penta-His\textsuperscript{®} mouse IgG, monoclonal antibody (anti-pentahistidine) <em>BSA free</em></td>
<td>100 µg</td>
</tr>
<tr>
<td>P-21856</td>
<td>Pro-O\textsuperscript{™} Emerald 300 Glycoprotein Blot Stain Kit <em>with SYPRO\textsuperscript{®} Ruby protein blot stain</em> <em>&gt;10 minigel blots</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>P-21857</td>
<td>Pro-O\textsuperscript{™} Emerald 300 Glycoprotein Gel and Blot Stain Kit <em>10 minigels or minigel blots</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>P-21855</td>
<td>Pro-O\textsuperscript{™} Emerald 300 Glycoprotein Gel Stain Kit <em>with SYPRO\textsuperscript{®} Ruby protein gel stain</em> <em>&gt;10 minigels</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>P-21874</td>
<td>Pro-O\textsuperscript{™} Emerald 488 Glycoprotein Blot Stain Kit <em>with SYPRO\textsuperscript{®} Ruby protein blot stain</em> <em>&gt;10 minigel blots</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>P-21875</td>
<td>Pro-O\textsuperscript{™} Emerald 488 Glycoprotein Gel and Blot Stain Kit <em>10 minigels or minigel blots</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>P-21873</td>
<td>Pro-O\textsuperscript{™} Emerald 488 Glycoprotein Gel Stain Kit <em>with SYPRO\textsuperscript{®} Ruby protein gel stain</em> <em>&gt;10 minigels</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>P-21851</td>
<td>Pro-O\textsuperscript{™} Fuchsia Glycoprotein Blot Stain Kit <em>10 minigel blots</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>P-21850</td>
<td>Pro-O\textsuperscript{™} Fuchsia Glycoprotein Gel Stain Kit <em>10 minigels</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>P-21870</td>
<td>Pro-O\textsuperscript{™} Glycoprotein Blot Stain Kit #1 <em>with concanavalin A and DDAO phosphate</em> <em>&gt;20 minigel blots</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>P-21853</td>
<td>Pro-O\textsuperscript{™} Glycoprotein Blot Stain Kit #2 <em>with concanavalin A, DDAO phosphate and SYPRO\textsuperscript{®} Ruby blot stain</em> <em>&gt;10-20 minigel blots</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>P-21871</td>
<td>Pro-O\textsuperscript{™} Glycoprotein Blot Stain Kit #3 <em>with wheat germ agglutinin and DDAO phosphate</em> <em>&gt;20 minigel blots</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>P-21854</td>
<td>Pro-O\textsuperscript{™} Glycoprotein Blot Stain Kit #4 <em>with wheat germ agglutinin, DDAO phosphate and SYPRO\textsuperscript{®} Ruby protein blot stain</em> <em>&gt;10-20 minigel blots</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>P-21872</td>
<td>Pro-O\textsuperscript{™} Glycoprotein Blot Stain Kit #5 <em>with <em>Griffonia simplicifolia</em> lectin II (GS-II) and DDAO phosphate</em> <em>&gt;20 minigel blots</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>P-21878</td>
<td>Pro-O\textsuperscript{™} Oligohistidine Blot Stain Kit #1 <em>with biotin NTA, streptavidin and DDAO phosphate</em> <em>20 minigel blots</em></td>
<td>1 kit</td>
</tr>
</tbody>
</table>

**References**

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**9.5 Reagents for Peptide Analysis, Sequencing and Synthesis**

This section describes Molecular Probes’ reagents used in the synthesis of fluorescent dye– or hapten-labeled peptides and fluorogenic protease substrates, as well as in peptide and protein sequencing. The dominant chemistry for sequencing peptides employs the nonfluorescent reagent, phenyl isothiocyanate, which forms phenylthiohydantoins (PTH) in the sequencing reaction. Some of our fluorescent probes and research chemicals have been used for N-terminal amino acid analysis and peptide sequencing, as well as for protein fragment modification prior to PTH sequencing.

**N-Terminal Amino Acid Analysis**

Except when it is already blocked by formylation, acetylation, pyroglutamic 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 (D-21) and dabsyl chloride (D-1537) are the most commonly employed reagents for such analyses.1–3

Nonacylated N-terminal serine and threonine residues of proteins can be peridate-oxidized to aldehydes1–6 (Figure 3.1) that can then be modified by a variety of hydrazine derivatives listed in Section 3.2. 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 (D-100) and subsequent chromatographic separation of the fluorescent product.7,8

The sensitivity of this method can likely be improved by the use of other fluorescent hydrazine derivatives described in Section 3.2.

**Peptide Sequencing**

As analogs of phenyl isothiocyanate, the peptide conjugates of fluorescein-5-isothiocyanate (FITC, F-143, F-1906, F-1907; Section 1.5), and other fluorescent isothiocyanates are susceptible to Edman degradation via their thiohydantoins. Thus, these fluorescent reagents are potentially useful for ultrasensitive amino acid sequencing.9–12

**Peptide Synthesis**

Peptides specifically labeled with fluorescent dyes, haptons, photoactive 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. Molecular Probes offers some 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 in Chapters 1–5 (see Labeling Small Peptides with Amine-Reactive Dyes in Organic Solvents). 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 the peptide’s thiol group by one of the thiol-reactive reagents described in Chapter 2 is usually easy, selective and very efficient.