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. However, with the rapid growth of proteomics [1] (Figure 9.1), 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 Multiplexed Proteomics.

Molecular Probes' Detection Technology for Proteomics

Molecular Probes is meeting the demands of the rapidly expanding field of proteomics through the development of fluorescence- and luminescence-based detection methods for proteins in solutions, in gels and on blots and microarrays. Several seminal papers describe our novel technologies and their applications (see Seminal Articles Using Molecular Probes' Luminescence-Based Protein Detection Technologies below). We are continuing to develop new reagents and detection methods for proteins and their modifications, such as glycosylation, phosphorylation 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, automatability and accurate quantitation capabilities. Application of our unique detection reagents requires minimal investment in labor, as compared to older technologies, while significantly increasing throughput, reducing total cost and accelerating discovery. Furthermore, the greater sensitivity and linearity of most of our premiere reagents makes it possible to do quantitative proteomics and perform comparative protein expression measurements on very small samples.

Multiplexed Proteomics

Fluorescence- or luminescence-based detection technologies also offer the opportunity for multicolor labeling, making multiplex analysis possible (Figure 9.2). In particular, Molecular Probes' technologies create the ability to identify specific proteins within the context of the entire protein profile. This capability, which we term Multiplexed Proteomics (MP), uses our luminescence-based total protein stains together with our novel fluorescence-based technologies for detection of specific proteins and protein modifications (for example, glycosylation, phosphorylation and epitope tags). 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 Multiplexed

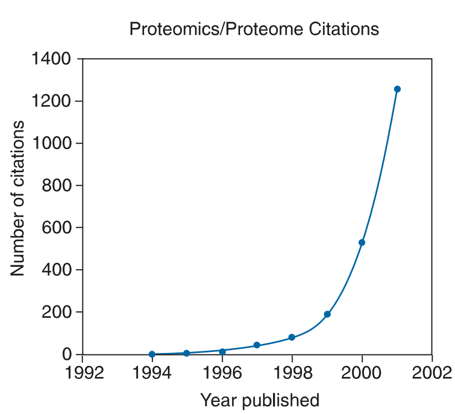

Figure 9.1 Growth of proteomics. Using the search parameter "proteom*" the NCBI database was queried for citations. The number of citations in each year plotted against the year shows the tremendous increase in research and interest in the proteomics field.

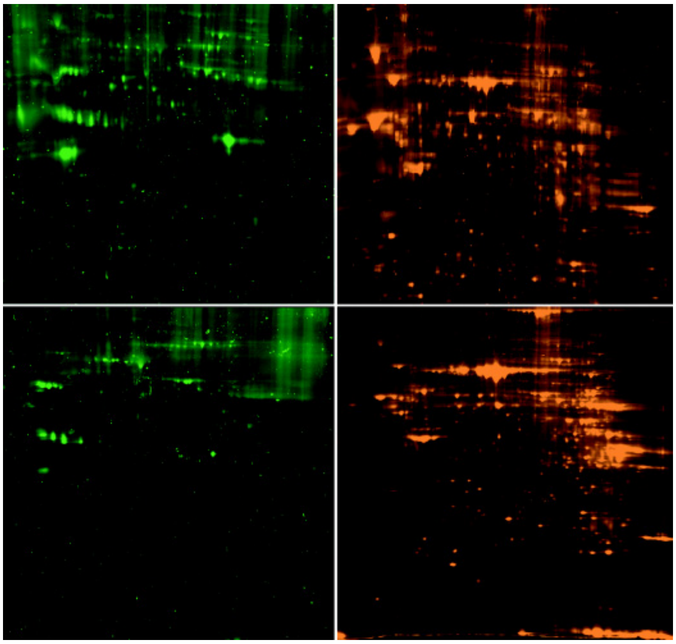

Figure 9.2 2-D protein gels of tumor vs. normal cells stained for glycoproteins and total proteins. Lysates from rat liver tumor cells (top panels) or rat normal liver cells (bottom panels) were run on identical 2-D gels. Following electrophoresis, the gels were stained with the Pro-Q Emerald 300 glycoprotein detection reagent (left panels) (available in the Pro-Q Emerald 300 Kits P-21855 and P-21857). After documentation of the fluorescence signal, the gel was stained with the SYPRO Ruby protein gel stain (right panels) (S-12000, S-12001, S-21900).

TECHNICAL NOTE

Seminal Articles Using Molecular Probes' Luminescence-Based Protein Detection Technologies

Reviews

"Making blind robots see: The synergy between fluorescent dyes and imaging devices in automated proteomics." W.F. Patton, Biotechniques 28, 944 (2000).

"A thousand points of light: the application of fluorescence detection technologies to two-dimensional gel electrophoresis and proteomics." W.F. Patton, Electrophoresis 21, 1123 (2000).

"Better approaches to finding the needle in a haystack: Optimizing proteome analysis through automation." M.F. Lopez, Electrophoresis 21, 1082 (2000).

SYPRO Ruby Protein Gel Stain

"Comparison of three different fluorescent visualization strategies for detecting *Escherichia coli* ATP synthase subunits after sodium dodecyl sulfate-polyacrylamide gel electrophoresis." K.N. Berggren et al., Proteomics 1, 54 (2001).

"Mass spectrometry compatibility of two-dimensional gel protein stains." W.M. Lauber et al., Electrophoresis 22, 906 (2001).

"Postelectrophoretic staining of proteins separated by two-dimensional gel electrophoresis using SYPRO dyes." J.X. Yan et al., Electrophoresis 21, 3657 (2000).

"A comparison of silver stain and SYPRO Ruby protein gel stain with respect to protein detection in two-dimensional gels and identification by peptide mass profiling." M.F. Lopez et al., Electrophoresis 21, 3673 (2000).

"Background-free, high sensitivity staining of proteins in one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gels using a luminescent ruthenium complex." K. Berggren et al., Electrophoresis 21, 2509 (2000).

"The current state of two-dimensional electrophoresis with immobilized pH gradients." A. Görg et al., Electrophoresis 21, 1037 (2000).

"A novel subfractionation approach for mitochondrial proteins, a three-dimensional mitochondrial proteome map." B.J. Hanson et al., Electrophoresis 22, 950 (2001).

SYPRO Ruby and SYPRO Rose Plus Protein Blot Stains

"A luminescent ruthenium complex for ultrasensitive detection of proteins immobilized on membrane supports." K. Berggren et al., Anal Biochem 276, 129 (1999).

"Herp, a new ubiquitin-like membrane protein induced by endoplasmic reticulum stress." K. Kokame et al., J Biol Chem 275, 32846 (2000).

"An improved, luminescent europium-based stain for detection of electroblotted proteins on nitrocellulose or polyvinylidene difluoride membranes." C. Kemper et al., Electrophoresis 22, 881 (2001).

SYPRO Orange and SYPRO Red Protein Gel Stains

"Analysis of tear protein patterns of dry-eye patients using fluorescent staining dyes and two-dimensional quantification algorithms." F.H. Grus, P. Sabuncuo, A.J. Augustin, Electrophoresis 22, 1845 (2001).

"SYPRO Orange and SYPRO Red protein gel stains: One-step fluorescent staining of denaturing gels for detection of nanogram levels of protein." T.H. Steinberg et al., Anal Biochem 239, 223 (1996).

"Applications of SYPRO Orange and SYPRO Red protein gel stains." T.H. Steinberg, R.P. Haugland, V.L. Singer, Anal Biochem 239, 238 (1996).

"Optimal filter combinations for photographing SYPRO Orange or SYPRO Red dye-stained gels." T.H. Steinberg, H.M. White, V.L. Singer, Anal Biochem 248, 168 (1997).

"Green-light transilluminator for the detection without photodamage of proteins and DNA labeled with different fluorescent dyes." F.J. Alba, A. Bermudez, J.R. Daban, Electrophoresis 22, 399 (2001).

SYPRO Tangerine Protein Gel Stain

"Fluorescence detection of proteins in sodium dodecyl sulfate-polyacrylamide gels using environmentally benign, nonfixative, saline solution." T.H. Steinberg et al., Electrophoresis 21, 497 (2000).

"Simultaneous, two-color fluorescence detection of total protein profiles and β-glucuronidase activity in polyacrylamide gel." C. Kemper et al., Electrophoresis 22, 970 (2001).

Pro-Q Emerald Glycoprotein Stain

"Rapid and simple single nanogram detection of glycoproteins in polyacrylamide gels and on electroblots." T.H. Steinberg et al, Proteomics 1, 841 (2001).

DyeChrome Western Blot Stain Kits

"A novel subfractionation approach for mitochondrial proteins: A three-dimensional mitochondrial proteome map." B.J. Hanson et al., Electrophoresis 22, 950 (2001).

"Green/red dual fluorescence detection of total protein and alkaline phosphate-conjugated probes on blotting membranes." K.P. Top et al., Electrophoresis 22, 896 (2001).

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

Molecular Probes' Reagents and Kits for Proteomics

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

- DyeChrome Western Blot Stain Kits for simultaneous dichromatic staining of total proteins and specific proteins on blots (and probably microarrays)
- DyeChrome Double Western Blot Stain Kit (D-21887) — the first technology that permits simultaneous trichromatic detection of total proteins and two different specific proteins or protein modifications on a single Western blot
- Pro-Q Western Blot Stain Kits for staining of proteins on blots using alkaline phosphatase–conjugated reagents and DDAO phosphate
- Pro-Q Emerald 300 and Pro-Q Emerald 488 glycoprotein gel and blot stains — the world's best and easiest-to-use stains for detecting periodate-oxidized glycoproteins
- Amplex Gold Kits for Western Blots, for staining of horseradish peroxidase–labeled targets on Western blots
- Pro-Q Sapphire 365 and Pro-Q Sapphire 488 oligohistidine gel stains, for fast and easy detection of oligohistidine fusion proteins in gels
- The BOLD APB chemiluminescent substrate (B-21901) for ultrasensitive detection of alkaline phosphatase conjugates on PVDF or nitrocellulose membranes

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

References

1. Proteomics 1, 169 (2001).

9.2 Quantitation and Selective Purification of Proteins in Solution

Several colorimetric methods have been described for quantitating proteins in solution, including the widely used Bradford[1] and Lowry[2] assays, as well as an assay described by Smith[3] that uses bicinchoninic acid (BCA). However, because they rely on absorption-based measurements, these methods are inherently limited in sensitivity and effective range. Molecular Probes has developed two unique fluorometric methods for quantitating proteins in solution — the NanoOrange Protein Quantitation Kit (N-6666) and the CBQCA Protein Quantitation Kit (C-6667) — that outperform *all* existing methods (Table 9.1). We also offer several other fluorescent reagents useful for protein detection in solution.

NanoOrange Protein Quantitation Kit

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

- **Ease of use.** The NanoOrange assay protocol is much easier to perform than the Lowry method (Figure 9.3). Protein samples are simply added to the diluted NanoOrange reagent, and the mixtures are heated at 95°C for 10 minutes. After cooling the mixtures to room temperature, their fluorescence emissions are measured directly. The interaction of proteins with the NanoOrange reagent produces a large fluorescence enhancement that may be used to generate a standard curve for protein determination; fluorescence of the reagent in the absence of proteins is negligible.
- **Sensitivity and effective range.** The NanoOrange assay can detect proteins at a final concentration as low as 10 ng/mL when a standard spectrofluorometer or minifluorometer is used. A single protocol is suitable for quantitating protein concentrations between 10 ng/mL and 10 µg/mL — an effective range of three orders of magnitude (Figure 9.4).
- **Stability.** The NanoOrange reagent and its protein complex have high chemical stability. In contrast to the Bradford and BCA assays, readings can be taken for up to six hours after sample preparation with no loss in signal, provided that samples are protected from light.
- **Little protein-to-protein variability** (Figure 9.5). The NanoOrange assay is not only more sensitive, but shows less protein-to-protein variability than Bradford assays.
- **Insensitivity to sample contaminants.** Unlike the Lowry and BCA assays, the NanoOrange assay is compatible with the presence of reducing agents. Furthermore, the high sensitivity of the assay and stability of the protein–dye complex make it possible to dilute out most potential contaminants, including detergents and salts (Table 9.2). Nucleic acids do not interfere with protein quantitation using the NanoOrange reagent. Although unusually high concentrations of lipids in the sample can interfere with the NanoOrange assay, this interference can